Functional Selection of Maize (*Zea mays* L.) Rhizobacteria Antagonizing *Fusarium graminearum*

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DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Science, Agriculture and Technology, School of Environmental and Health Sciences, and the work contained herein is my original work with exception of the citations and that this work has not been submitted at any other University in part or entirety for the award of any degree.

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DEDICATION

This work is dedicated to the Almighty God for life given, wisdom, understanding, knowledge, insight, inspiration and illumination imparted and to my parents the pioneers of my achievements.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL ABSTRACT

This study was designed to select for indigenous prospective biosuppressors of *F. graminearum* from the popular bacterial genera *Bacillus* and *Pseudomonas* that will become environmental friendly alternatives for maize protection. Through *in vitro* chromogenic and molecular techniques, 400 bacteria isolates were selected from the maize rhizosphere of ten farms in South Africa's North West Province, however only 3.5% showed acceptable antifungal potentials for further studies. Each genera had 7 selected isolates to which lipopeptide antibiotics responsible for antifungal potentials were attributed. The pseudomonads harbored gene clusters for the secretion of antibiotics including, pyrrolnitrin, HCN, 2, 4-DAPG diacetlyphloroglucinol and phenazine while *Bacillus* spp. had genes responsible for the synthesis of iturin, bacillomycin, surfactin and fengycin, and these were detected through PCR amplification. The 2 consistent isolates (*Bacillus* sp. BS10.5 and *Pseudomonas* sp. PS9.1) showing high *in vitro* inhibitory potentials against *F. graminearum* and *F. culmorum* with biosurfactant production capability were selected for further studies to determine their bioprotective ability against seed borne and root fusariosis. During *in vitro* seed assay, the mean enhanced seed germination and reduction of seed borne incidence of fusariosis by the two strains was > 50% and > 60% respectively. Pot experiments conducted in sterile and unsterilized soil during three experimental periods to determine the stability of BS10.5 and PS9.1 for field studies, revealed their effectiveness at protecting maize during germination. Overall, the percentage suppression of *F. graminearum* aggression by the isolates was higher in sterile soils. Despite the increased dose level of the fungal pathogen treatment (10^5 spores ml^{-1}) during the third experimental period, the treatments with the antagonists performed better than the control which wilted and died off. The root dip pre-sowing method employed for seed bacterization was effective at protecting maize seedling germination up to VT stage of growth. The two isolates were effective in reducing a *Fusarium* infection of maize seedlings. Also, treatment of seeds with

PS9.1 and BS10.5 resulted in increased germination rate of seeds. This bioprotective effect given by the antagonists was seen from the growth parameters taken from both pathogen and non-pathogen treated plants. Considering the environmental survivability of the *Bacillus* spp., which is due to their endospore forming capability, we evaluated the antimicrobial potentials of the secondary metabolite secreted by *Bacillus* BS10.5. Having seen that the cell free substances showed potent anti-phytopathogenic, we further characterized its lyophilized lipopeptide extracts. Because microbial secondary metabolites contain diverse constituents that often overlap and create difficulty during purification and identification, we combined multiple analytical procedures for the structural elucidation and chemical characterization of the compounds, which revealed the presence of the notable cyclic lipopeptides. The NMR, FTIR and ESI-MS analysis of the lyophilized extracts showed the presence of notable cyclic lipopeptides iturin, surfactin, bacilomycin (m/z 1058.6738, 1058.6740) and fengycin (m/z 1477.8184) which have been proven to exhibit broad spectrum antimicrobial properties relevant to crop protection. The bioprotective capability of the BS10.5 strain was further proven when we mined its genome *in silico*. BS10.5 had a minimum of 16 biosynthetic clusters of gene dedicated to the synthesis of PKS, NRPS and other peptides. The data from the metabolic modelling of the genome unveiled 28 previously identified compounds, 1558 reactions, 1559 compounds, 1000 genes and additionally gave credence to the experimental data gathered *in vitro* showing the geno-taxonomic affiliations and biosuppressive potential of this native *Bacillus* isolate against members of the *Fusarium* spp. especially *F. graminearum*. This study reveals how indigenous rhizobacterial organisms are involved in host plant protection.

DISSEMINATION OF RESEARCH RESULT AND LIST OF PUBLICATIONS

A. Presentation (Conference proceeding) at Society for Industrial Microbiology and Biotechnology Annual Meeting and Exhibition, Denver (CO), USA, 30th July – 3rd August, 2017 (see appendix).

B. Presentation (Conference proceeding) at Society for Applied Microbiology Conference and Exhibition, Edinburgh, UK, 4th – 7th July, 2016 (see appendix).

Chapter 2: Tackling Maize Fusariosis: In Search of *Fusarium graminearum* Biosuppressors.
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Candidate's Contributions: managed the literature searches, did all the wet laboratory bench work, performed all the analyses, interpreted the results and wrote the first draft of the manuscript.

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LIST OF ABBREVIATIONS

LB.....Luria Bertani

rpm.....Revolutions Per Minute

Fcul.....*Fusarium culmorum*

Fg.....*Fusarium graminearum*

KP.....*Klebsiella pneumonia*

MC.....*Moxarella cartarrhalis*

BC.....*Bacillus cereus*

EF.....*Enterococcus faecalis*

PA.....*Pseudomonas aeruginosa*

BGC.....Biosynthetic Gene Cluster

Fig.....Figure

Min.....Minute

L.....Liter

Kbase.....DOE Systems Biology Knowledgebase

µl.....Microliter

µm.....Micrometer

µg.....Microgram

CFU.....Colony Forming Unit

bp.....Base pair

NCBI.....National Center for Biological Information

BLAST.....Basic Local Alignment Search Tool

PDA.....Potato Dextrose Agar

OD.....Optical Density

BCA.....Biological Control Agent

Hr.....Hours

%.....Percentage

spp.....Species (plural)

sp.....Specie (singular)

PRISM.....Prediction Informatics for Secondary Metabolomes

NRPS.....Non Ribosomal Peptide Synthetase

trans–AT.....Trans-Acyl Transferase

PKS.....Polyketide Synthase

T3pks.....Type 3 polyketide synthetase

Ks.....Ketosynthase

KEGG.....Kyoto Encyclopedia of Genes and Genomes

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction to this chapter

One of the fundamental goals of both developing and developed nations is making sure poverty levels are alleviated and food is made available to the global population. Staple crops such as wheat, barley, rice and maize remain first choice foods globally. However, plant pathogens and the diseases they cause in these plants still incur great economic loss in the production and availability of these crops (Goyal *et al.*, 2014). Maize is one of the oldest cultivated crop world-wide and it is considered a key ingredient in animal feed, human dietary constituents and industrial raw materials in South Africa (Boutigny *et al.*, 2011) and many regions all over the world (Rosas *et al.*, 2009) with a production of 12,486, 000 tons in South Africa in 2013 (FAOSTAT, 2013).

Due to emerging and re-emerging diseases of crops worldwide, maize still remains one of the most studied of the plant species. Fungal diseases are among the most notable causes of crop loss in the world and significant increases in fungal infection in small grain cereals such as maize have been reported worldwide (Hernandez-Leon *et al.*, 2015). *Fusarium* species are widely distributed and are amongst the most frequently isolated causative agents of mycoses in maize in the field as well as during storage by plant pathologists (Kazan *et al.*, 2012).

Maize availability suffers greatly from infection by these fungi due to contamination of grain with mycotoxins rendering the grain unsuitable for human consumption, livestock use and industrial processing (Morcia *et al.*, 2013). Fumonisin and tricothecenes are

mycotoxigenic compounds secreted by members of the *Fusarium* genus and their presence in cereal grains poses a public health threat (Boutigny *et al.*, 2011). Recent research has reported that *Fusarium graminearum* is not a single species but a complex comprising of 16 distinct lineages now known as the *F. graminearum* species complex (FGSC). From the complex, five species namely *F. meridionale*, *F. asiaticum*, *F. austroamericanum, F. boothii*, and *F. graminearum sensu stricto* have been interconnected with maize diseases in South Africa with *F. boothii* being the most virulent (van der Lee *et al.*, 2014).

In South Africa, maize is cultivated during the late spring or early summer months with ideal or optimal planting periods in November and December. The major planting regions are the North West, Free State and Mpumalanga Provinces of South Africa. Depending on climatic factors and weather conditions, planting sometimes starts early October and extend to January, while harvest takes place from late May to August ending. The maize production systems in place include subsistence farming, small scale and large scale commercial farming with white maize (52%) being more widely cultivated than yellow maize (48%). A few reports have shown that two fungicides tebuconazole and metconazole, of the triazole family can control DON and FHB contamination in wheat. However, till date, no fungicide has been registered for cereal/grain fusariosis control in South Africa (Beukes et al., 2016). Because cereal ears are covered with tight husks, fungicides applied to control mycotoxigenic *Fusarium* spp. in maize cannot penetrate and are ineffective. The husks prevent contact with the pathogens, as a result of this, the use of resistant cultivars has been the most utilized approach to reduce fusariosis in cereals (Doohan et al., 2003; Xu *et al.*, 2011).

To obtain high productivity in most cultivated crops, including maize, it is necessary to carry out crop management practices that do not adversely affect the environment. Application of traditional mineral fertilizers to the soil is one of the most expensive agriculture practices and which also causes imbalance in natural ecosystems (Das *et al.*, 2013). Resistant cultivars, chemical fungicides and pesticides, and crop rotation have been the main strategies for controlling *Fusarium* diseases despite the observed variability in their effectiveness (Yuen and Schoneweis 2007; Xu *et al.*, 2011). The increased public health concern of environmental pollution attributed to the use of fungicide and pesticide residues, including the highly reported pathogen resistance to some pesticides, motivates plant pathologists, ecologists and consumers to find alternative methods for disease management and plant protection.

For several decades, biological control of phytopathogens using antagonistic microbes has become the leading, sustainable, safe and environmentally friendly method of disease management and plant protection (Lugtenberg and Kamilova 2009; Berendsen *et al.*, 2012) and the investigation using PGPR has been on for over a century (Lugtenberg and Kamilova 2009; Mitter *et al.*, 2013). Despite this long period of study, biological control laboratory strains that have high potential and promising tendencies still face insurmountable obstacles to commercialization.

With the advancement of molecular techniques, several novel rapid assays have been developed that have enabled the rapid detection and identification of specific bacterial strains capable of secreting beneficial metabolites for plant growth promotion, and it remains an area to be exploited. These secreted metabolites, also called allelochemicals, with examples like lipopeptides (Weller, 2007), have been identified, purified and quantified for large scale

production and use in various agricultural field practices (Babalola *et al.*, 2002; Frapolli *et al.*, 2007; Ramarathnam *et al.*, 2007; El-Sayed *et al.*, 2008; Von Felten *et al.*, 2011). The study of the genetic diversity of these plant associated microbial communities secreting beneficial metabolites, using numerous culture dependent and culture-independent approaches, specifically targeting genera such as *Pseudomonas* spp. and *Bacillus* spp. in the rhizosphere and endosphere of crop plants, have been conducted and reported (Kloepper *et al.*, 2004; Frapolli *et al.*, 2008; Dimkic *et al.*, 2013; Hernandez-Leon *et al.*, 2015).

The different approaches to studying genetic diversity available for ecological studies are based on PCR amplification protocols with varying primer sets (von Felten *et al.*, 2011). In recent years, systems developed to assess the diversity of specific microbial groups by using genus-specific and functional genes specific primers sets for rapid screening of plant growth promoting bacteria have been employed (Garbeva *et al.*, 2001; Bergsma *et al.*, 2005a; Bergsma *et al.*, 2005b; Costa *et al.*, 2006; Ramarathnam *et al.*, 2007; De La Fuente *et al.*, 2008; Moynihan *et al.*, 2009). This has become a new area of interest (Cordero *et al.*, 2012; Dunlap *et al.*, 2013; Kim *et al.* 2013), opening up exciting possibilities for the study of gene expression of microbes in environmental samples.

The ascomycetes *Fusarium graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schw.) Petch] causes fusariosis with different symptoms (ear rot, root rot, leaf rot) in maize, resulting in poor grain yield and accumulation of fungal mycotoxins (deoxynivalenol (DON) and zearalenone) in the grain (Wang *et al.*, 2011). For example, *F. graminearum* enters the maize through the silk-channel for ear rot infections and also enters maize ears through injuries inflicted on kernels by insects or birds (Sutton *et al.*, 1982; Zhang *et al.*, 2012). Earlier studies showed that the acuteness of ear rot symptoms increases during cool

temperatures (under 23°C), accompanied by rainfall, and that only *F. graminearum* secrets DON under wet conditions (Doohan et al., 2003) (Table 1.1).

Table 1.1: The mycotoxins secreted by *F. graminearum*, optimal conditions for production on the popular cereals and health threat posed.

Toxin	Substrate	Optimum production conditions	Health threat	References
ZEA	Maize , rice and wheat	Warm (17–28 ◦C), or temperature cycles (e.g. 25–28 ◦C for 14–15 days; 12–15 ◦C for 20–28 days) and humid (*a*w = 0.97 or 90% RH)	**Humans and livestock**: reproductive disorders, Hypo-estrogenic syndromes and stimulates the growth of breast cancer cells	´Lori et al. (1990), Jimenez et al. (1996), Ryu and Bullerman (1999), Homdork et al. (2000), Martins and Martins (2002)
Type B trichothecenes (3-acetyl DON, 15-acetyl DON,	Maize, barley, rice and wheat	Warm and humid (25–28 ◦C, *a*w = 0.97)	**Humans**: nausea, vomiting, diarrhea and other gastrointestinal illness; **Livestock**:	Sutton et al. (1982) Greenhalgh et al. (1983), Lori et al. (1990),

nivalenol (NIV), deoxynivalenol (DON),	food refusal, vomiting, decreased weight gain, anorexia, decreased feed consumption and decreased liver weights	Beattie et al. (1998), Homdork et al. (2000)

Studies on the biological control of *F. graminearum* have been promising recently due to the positive application of several biocontrol agents (Shi *et al.*, 2014; Zhao *et al.*, 2014). PGPR with multiple biocontrol capability have been shown to reduce growers' dependency on synthetic chemicals, which negates the overall development of antimicrobial resistance in pathogen populations (Jochum *et al.*, 2006; Crane *et al.*, 2013). Despite the numerous microbial strains reported to have potential antagonistic effects against *F. graminearum*, the genera *Pseudomonas* and *Bacillus* appear frequently in articles (Abdulkareem *et al.*, 2014; Martinez-Absalon *et al.*, 2014; Hernandez-Leon *et al.*, 2015).

Pseudomonas spp. remains one of the most significant groups contributing to production of beneficial antimicrobial compounds used in plant disease management (Weller *et al.*, 2007; Rosas *et al.*, 2009; Kim *et al.*, 2013). Culture based techniques along with advance molecular based approaches have been utilized in selecting diverse groups of plant growth promoting *Pseudomonas* (De La Fuente *et al.*, 2006). Individual *Pseudomonas*

strains, despite the reported low shelf life they exhibit during processing for commercialization, storage and field applications, still remain strong candidates for biocontrol studies (Kumar *et al.*, 2007; Rosas *et al.*, 2009; Calderon *et al.*, 2014) because they are active colonizers of the rhizosphere. In contrast, *Bacillus* strains have shown great advantage over other biocontrol microorganisms due to their capability of forming endospores that allow them to survive for extended periods under unfavorable environmental conditions. This is significant in their biocontrol and plant growth promoting potential (Zhang *et al.*, 2006; Dimkic *et al.*, 2013).

1.1.1 Rhizosphere Bacteria

The rhizosphere is the soil region surrounding plant roots in which roots and soil microbes interact directly and/or indirectly. It supports a diverse, densely populated microbial community and is subjected to chemical transformations caused by the root exudates and metabolites of microbial degradation (Van der Putten 2010 and Mendes et al., 2013). Most of these microorganisms living in this region have insignificant effects on plant growth, but others play crucial roles in plant nutrition, growth promotion and disease interactions. Reports documenting the relevance of plant growth promoting strains from the genera *Pseudomonas* and *Bacillus* (PGPP and PGPB) isolated from the rhizosphere widely exist (Raaijmakers et al., 2006; Ongena and Jacques 2007; Babalola, 2010; Dimkic *et al.*, 2013).

1.1.2 Microbial Secondary Metabolites Identification and Characterisation

Microbial secondary metabolites are bioactive compounds that include siderophores, antibiotics, volatile metabolites, and enzymes synthesised by various plant growth promoting bacteria, that are of vital importance in plant disease management (Sturz and Christie, 2003;

Van der Putten 2010; Saraf *et al.*, 2014). In addition to their use as alternatives to synthetic agrochemicals (Sturz and Christie, 2003; Farooq *et al.*, 2011), microbial metabolites may function to protect hosts against fungal suppression by inducing the up-regulation of pathogenesis-related genes of host plants (Chen *et al.*, 2006; Dunlap *et al.*, 2011; Chen *et al.*, 2015).

Cell free supernatant secondary metabolites (allelochemicals) extracted from PGPR strains using sensitive, specific and rapid detection techniques have been tested in greenhouse and field experiments (Wen *et al.*, 2011; Cordero *et al.*, 2012; Goswami *et al.*, 2015; Zhang *et al.*, 2015) and then produced for industrial and commercial application (Schisler *et al.*, 2002a; Schisler *et al.*, 2002b; Dimkic *et al.*, 2013; Hernandez-Leon *et al.*, 2015). The ability to identify gene functionality (Dunlap *et al.*, 2013) and rapidly quantify the active antifungal metabolites in several strains of the two genera (*Pseudomonas* and *Bacillus*) (Phister *et al.*, 2004; Ramarathnam *et al.*, 2007; Wang *et al.*, 2007; Yuan *et al.*, 2012) has allowed the industrial large scale production of biopesticides, biofertilizers and biosuppressors.

Numerous chromatographic methods coupled with spectrometric techniques are available for the characterization of microbial secondary metabolites, some of which are high performance liquid chromatography (HPLC), liquid chromatography electrospray ionization mass spectrometry (MS) technique (LC-ESI-MS) Nuclear Magnetic Resonance (NMR), Gas chromatography (GC), as well as Fourier Transform Infrared Spectroscopy (FTIR). These highly reproducible analytic methods when utilized together, enable detection, quantification and structural elucidation of the active components secreted by candidate biocontrol organisms (Yuan *et al.*, 2011; Ziegler *et al.*, 2014; Deepak and Jayapradha *et al.*, 2015; Zhang *et al.* 2015).

1.1.3 Genome mining

Genome mining has revolutionized the natural product discovery and biological control industry. Unraveling the total genomic properties and capabilities of beneficial bacteria has recently led to identification of cryptic and novel antimicrobial compounds (Challis, 2008; Ziemert et al., 2016). It has enhanced current knowledge on the biosynthetic repertoire of bioactive compounds of many biocontrol, plant-associated and plant growth promoter strains (Challis, 2008; Dunlap et al., 2014). Researchers have performed detailed *in silico* analyses of sequenced genomes and utilized the information retrieved in detecting, characterizing and producing compounds otherwise difficult to isolate or characterize during in vitro experiments (Kreutzer and Nett 2012; Loper et al., 2012; Michelsen et al., 2015). The combination of multiple techniques such as *in vitro* agar plate assays, greenhouse experiments, molecular genetics, genomics, various chromatographic and mass spectrometry analysis have been used to show the biocontrol ability of bacteria species and the metabolic genes they harbour (Michelsen *et al.*, 2015; Hertlein et al., 2016).

1.2 Problem statement

Fusarium infection of cereal grains is of public health and economic importance in South Africa where maize is a staple crop. Despite several attempts made to manage maize diseases caused by *Fusarium graminearum*, such as the Fusarium head blight (FHB), Seedling blight and Fusarium Ear Rot of maize (FER), there still exists a dearth of reports on the control of maize fusariosis. The continued incidence of maize fusariosis thus necessitates a strategic intervention. As reported by Boutigny *et al.*, (2012), changes in the spread and incidence of major *Fusarium* spp. interconnected with maize in South Africa showed a remarkable increase in *F. graminearum* infection in the past 20 years. In light of this,

assumptions cannot be made that the interaction between other *Fusarium* spp. infecting maize and their biocontrol counterparts is similar in infections of *F. graminearum*. The recent discovery of the *Fusarium graminearum* species complex (FGSC) calls for urgent intervention.

Reports showing the activity of PGPR to suppress the deleterious effect of *F. graminearum* on maize in Africa are wanting; the information on *F. graminearum* infection such as *Gibberella* ear rot and its potential biocontrol agents cannot be extrapolated from research reports documented for the control of other species of fusarium infecting maize from other geographic regions of the world (Babalola, 2010; Small *et al.*, 2012; Wagacha *et al.*, 2012). The reports showing the biosuppressive capabilities of indigenous biological control agents against endemic cereal pathogens is lacking in most African countries.

1.3 Justification of the study

The majority of the research done to tackle fusariosis in cereals has been carried out on wheat and barley and there has been little focus on *F. graminearum* diseases. The negative effect of maize Fusariosis in South Africa needs urgent attention. It appears that *in vitro* studies, which sometimes show little correlation with greenhouse experiments or field trials, represent the bulk of reports available on management of fusariosis. Variances exist in the antagonistic potential exhibited by commercial biocontrol strains during plant disease management when they are applied in geographic regions outside their origins of isolation. Although reports on the activity of commercialized biocontrol strains from the genera *Bacillus* and *Pseudomonas* widely exist, there is a need to further understand and explore the genomic potential of indigenously isolated strains from both genera for geographical stability, due to their continued relevance.

1.4 General objective

This study was designed to provide an effective biocontrol *Pseudomonas* and *Bacillus* agent for the management of maize fusariosis caused by *F. graminearum*.

1.4.1 The specific objectives of this study were to:

1. Identify active indigenous strains of *Pseudomonas* and *Bacillus* from maize rhizosphere capable of suppressing the deleterious effect of *F. graminearum* on maize.
2. Determine genetic relatedness of the isolated strains.
3. Evaluate the stability of potentially selected indigenous *Pseudomonas* and *Bacillus* strains for consistent field application.
4. Determine the active metabolite detected in the selected microbial strains.
5. Understand the genomic potential of the best isolate identified.

1.4.2 Significance of the study

This study should provide a better understanding of the complex interactions that exist between PGPB, *F. graminearum* and maize crops. The benefits of applying metabolomics based approach to identifying the potential of biocontrol agents will be emphasized and the evidences obtained from *in vitro* and *in vivo* biocontrol assays carried out in this study should provide maize growers with an alternative disease control strategy that will encourage crop management practices that do not pose a public health threat in South Africa.

1.5 Research questions

- Can indigenous rhizopheric strains from the genera *Pseudomonas* and *Bacillus* suppress *F. graminearum* infection in maize?
- Do native maize rhizopheric strains from the genera *Pseudomonas* and *Bacillus* harbour biosynthetic genes responsible for synthesizing antimicrobial metabolites?
- Do rhizopheric strains from the genera *Pseudomonas* and *Bacillus* suppressing *F. graminearum* secrete secondary metabolites that can be used in biocontrol processes?
- Can these indigenous strains be of benefit for other plant disease management strategies?

CHAPTER TWO

TACKLING MAIZE FUSARIOSIS: IN SEARCH OF *FUSARIUM GRAMINEARUM* BIOSUPPRESSORS

Abstract

This review presents biocontrol agents employed to alleviate the deleterious effect of the pathogen *Fusarium graminearum* on maize. The control of this mycotoxigenic phytopathogen remains elusive despite the elaborate research conducted on its detection, identification and molecular fingerprinting. The majority of research done to tackle *F. graminearum* outbreak are on wheat and barley. Variances also exist in the antagonistic potential of biocontrol strains on *F. graminearum* in diverse cereal grains and their cultivars. This review also reveals that *in vitro* and greenhouse biocontrol studies on *F. graminearum* exceed the number of field studies. Biocontrol strains from the genera *Bacillus* and *Pseudomonas* appear frequently in controlled experiments carried out to improve maize production and most fusariosis management in maize has been on other members of *Fusarium* such as *Fusarium verticillioides*. We highlight relevant current techniques needed to identify an effective biofungicide for maize fusariosis and recommend alternative approaches to reduce the scarcity of data for maize field trials.

2.1 Introduction

Fungal pathogens pose a great challenge to grain production in several regions of the world. The threat is reported in many continents with the members of the *Fusarium* spp. still frequently encountered as causative agents of fusariosis. The dominant species of *Fusarium* that cause maize rots worldwide are *F. verticillioides*, *F. graminearum* and *F. culmorum*, *F. proliferatum*, and the more recent less significant species include *F. subglutinans*, *F. sporotrichioides* and *F. temperatum* (Summerell et al. 2011 and Czembor et al. 2015). Significant genetic and morphological diversity was observed within species associated with *F. graminearum* across geographic regions (Przemieniecki et al. 2014 and van der Lee 2015) and this prompted researchers to establish the *F. graminearum* species complex (FGSC lineages). Species within the FGSC cause head blight diseases and serious rots of several cereal crops, such as maize, barley and wheat world-wide (Sampietro et al. 2012; Yang et al. 2013; Suproniene et al. 2016). They are still responsible for the periodic epidemics of fusariosis that result in significant economic losses due to reduction in grain yield and quality.

Production of maize in developing countries is done on nearly 100 million hectares and 70% of the total maize produced in the developing world, where demand is expected to double by 2050, comes from countries with low and lower middle income (Cairns et al. 2012). Members of the FGSC such as *F. graminearum sensu stricto* belonging to lineage 7, (still commonly called *Giberrella zea*), secrete toxins that include nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) and the presence of these phytopathogens or their toxins in cereal grains poses a public health threat. The toxic effect of these mycotoxins secreted on animals and humans in several geographic regions globally is a cause for concern (Mullen et al. 2012 and Varga et al. 2015).

F. graminearum clade comprising at least 16 phylogenetically distinct species was divided into various species using nucleic acid based techniques (O'Donnell et al. 2004; Wang et al. 2011; Aoki et al. 2012). FGSC were identified based on evolutionary mechanisms and a simultaneous analysis of multiple sequences (loci) using diagnostic methods involving genealogical concordance phylogenetic species recognition (GCPSR) loci and multilocus genotyping assay (MLGT) loci (O'Donnell et al. 2004 and 2008). The GCPSR approach supports the determination of similarities and boundaries between fungal species while the MLGT method relies on an analysis of single nucleotide polymorphism (SNPs). Both methods generate a marker database used to monitor taxon migration, variances within a population, and the mycotoxin dispersal within species (Zhang et al. 2012).

Controlling the emergence of fusariosis or rots caused by *F. graminearum* on maize with chemicals has been difficult, largely due to the nature of the pathogen and the prevailing climatic conditions (Bacon et al. 2007). For example, *F. graminearum* enters the maize through the silk-channel for ear rot infections and also enters maize ears through injuries inflicted on kernels by insects or birds (Sutton et al., 1982 and Zhang et al. 2012). Earlier studies showed that the acuteness of ear rot symptoms increases during cool temperatures (below 23°C), accompanied by rainfall, and that only *F. graminearum* produces DON under wet conditions (Doohan et al., 2003) (Table 1).

The application of chemical fungicide to maize seedlings prior to planting has not been effective, rather it leads to significant increases in mycotoxin concentrations in plants (Pereira et al. 2009 and Small et al. 2012). The ascomycetes *F. graminearum* causes fusariosis with different symptoms (ear rot, root rot, leaf rot) in maize, resulting in poor grain yield and accumulation of fungal mycotoxins (DON, NIV and ZEA) in the grain (Wang et al. 2011).

The thorough study of the problem and effective control strategies of this disease to maize production are still necessary. Most research has tilted towards using biological control as an alternative for alleviating plant diseases against chemical control (Heydari and Pessarakli, 2010 and Babalola and Glicks, 2012), and large numbers of bacterial species predominantly *Pseudomonas* and *Bacillus* strains, have been frequently identified to be highly antagonistic against agents of fusariosis (Pérez-Montaño et al. 2014).

The most common approach utilized for biocontroller innovation chain was proposed by Bailey et al. (2010), and involves (a) screening and early discovery of strains, (b) proof of field applicability, (c) fermentation development procedures, (d) formulation and application into technological platforms, and lastly implementation into farming systems. Till date many of the studies do not pass the screening stages; few studies have identified or reported commercialized biocontrollers for FGSC. Often, laboratory assessment data that are temporary screening methods are the only readily available report, while field experimental studies are not readily available. Even when available most reports show no relationship between the reactions *in vitro* and *in planta*. Most of the earlier field trials were solely performed to identify management strategies for single mycotoxigenic fungus and its respective toxin (Chandra et al. 2009).

Several factors affect the efficacy of potential biocontrol agents in field experiments ranging from culture formulations, dosage of microbial inoculants, crop cultivars, experimental site, and changing weather conditions. The compatibility of a PGPR strain with commonly used fungicides, spermosphere and rhizosphere competence are pre-requisites for reproducible biological control activity during field studies. Reports involving field studies showing the successful use of an antagonist during plant disease management are not readily

available (Xu et al. 2009). *In planta* studies often give a realistic indication of the biocontrol measure achievable in real time environmental situation. This chapter discusses primarily the strategies used in finding biocontrol agents that are able to suppress maize fusariosis caused by *F. graminearum*. It further highlights the efforts made at providing biocontrollers for the management of *F. graminearum* maize fusariosis.

Screening approaches used for selecting *Fusarium graminearum* biocontrollers

Whatever the approach decided on in selecting for a BCA's against phytopathogens, it is important to decide on whether to eliminate toxin secretion, disease severity or maybe to reduce both the secretion of toxins and stop the onset of disease. Previous reports demonstrate that there is a positive linear relationship between the occurrence of fusariosis and toxin contamination (Wegulo et al. 2012, 2015). Kohl et al. (2011) concluded that most screening approaches that have been employed have focused on antagonistic efficacy shown by potential biological control strain during *in vitro* or greenhouse test, as the criteria for their selection. Many did not highlight other characteristics of the potential biocontrol strain that would be relevant for commercial exploitation during their screening approaches. Walsh et al. (2001) and Kohl et al. (2011), have proposed screening approaches and commercialization strategies that could be adopted for selection of BCAs. **Figure 2.1** describes the sequential events that takes place from the isolation stage of a potential biocontrol strain to its commercialization.

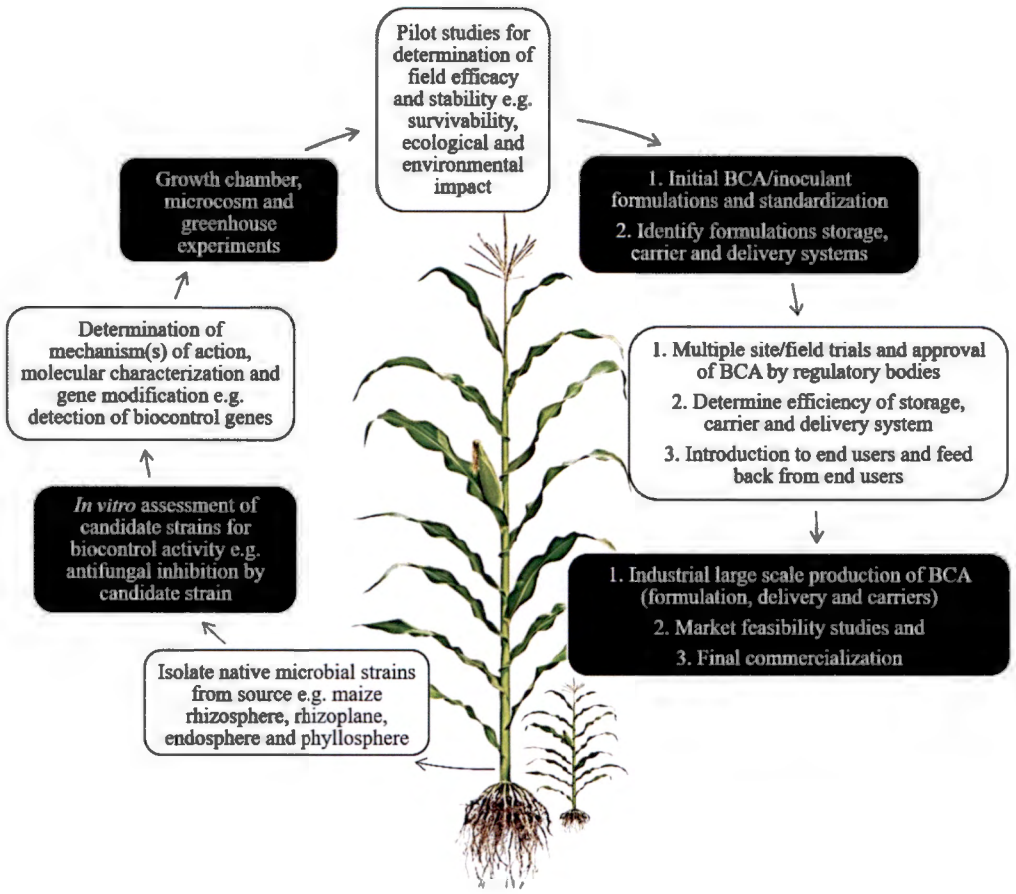


Figure 2.1: Flow diagram of the sequential events that takes place from the isolation stage of a potential biocontrol strain to its commercialization.

2.1.1 Potential biocontrol strains evaluated *in vitro*

The progression in determining the biocontrol potential of a rhizospheric isolate for the inhibition of fungal phytopathogens includes *in vitro* tests, such as dual culture agar plate test and tip culture assay. These assays range from using antagonists to inhibit growth of the pathogen or completely kill the pathogen to using their metabolites as inhibitors. The *in vitro* test are mostly used to select for the most effective isolates, which are then utilized in further plant bioassay conducted with crop seedlings. This initial step narrows down the total number

of microbial isolates that show presumptive antagonistic potential and the inhibition zones seen when the agar plates are used signify the rate of susceptibility of the pathogen.

The *in vitro* culture selection process is a necessary systematic, comprehensive method for high throughput screening of microbes for antifungal activity (Shehata et al. 2016a). Although laborious, the method captures the majority of the microbes both weak and strong that can suppress pathogen proliferation *in planta*. This provides strong evidence that a BCA performing greatly *in planta*, was firstly identified or chosen from a numerous pool of potential strains. However, the possibility of overlooking strains that could have strong field viability and efficacy but exhibiting weak *in vitro* antagonistic potential is not always considered (Schöneberg et al., 2015). *In vitro* tests could also include monitoring seed assays in petri dishes or conical flasks for a specific length of time for disease manifestation or improved plant growth. The selection process could be less laborious if differential and selective chromogenic detection methods that target specific genera are included in the isolation stages.

In vitro studies carried out by Abdulkareem et al. (2014) showed that *Azotobacter chroococcum*, *Bacillus pumilus, Azospirillum* sp., and *B. subtilis* isolated from the cucumber rhizosphere reduced the mycelia growth of *F. graminearum* in a dual culture test. *Azospirillum* sp produced volatile metabolites but the secretion was dependent on the growth phases. The antagonistic effect of *Azospirillum* sp. disappeared at day 7, however, that of *B. subtilis* continued after day 7. Dunlap et al. (2011) investigated the antagonistic potential of three *Bacillus* strains against *F. graminearum*. Two *Bacillus* strains (As.43.3, As.43.4) inhibiting *F. graminearum* and producing three lipopeptides, iturin, fengycin, surfactin were identified. A third strain OH131.1 producing only the lipopeptide surfactin showed no

inhibition when its culture free supernatant was used as antimicrobial substance suggesting that strain OH 131.1 utilized a different mechanism for its inhibition against *F. graminearum*.

Cordero et al. (2012) investigated the antagonistic potentials of several *Pseudomonas* strains isolated from the maize rhizosphere *in vitro* (growth media). *Pseudomonas* strain MGR39 inhibited *F. graminearum* due to production of hydrogen cyanide (HCN) and pyrrolnitrin detected biochemically and by polymerase chain reaction (PCR) amplification. Pagnussatt et al. (2014) evaluated the effects of crude and purified extracts of phenolic acids from *Spirulina* LEB-18 strain on the mycotoxin production, mycelial growth, enzyme activity and glucosamine content of some strains from the FGSC. The addition of the phenolic extracts to the *F. meridionale* and *F. graminearum* growth media caused reduction in the growth of the phytopathogens, affected their mycelia growth, glucosamine content and mycotoxin concentration.

Laslo et al. (2012), isolated 12 bacterial strains from the rhizosphere and isolated 35 from the bulk soil, and screened them for plant growth promoting activities with the aim of using them as bioinoculants. Testing them against *F. graminearum in vitro*, the result showed a large percentage of the isolates inhibiting *F. graminearum*. Of the 47 isolates, of which the majority were *Pseudomonas* and *Bacillus* sp., six isolates from the rhizosphere showed strong antifungal activity against *F. graminearum*. Seventeen of the total bacterial isolates produced siderophores while another 30 mobilized calcium and phosphate. Similarly, Wang et al. (2007) identified a soil strain of *B. subtilis* (IB) that inhibited *F. graminearum* using *in vitro* agar plate tests. The *Bacillus* strain produced Fengycin A, C16, which exhibited broad range fungal spectrum at different concentrations and the lipopeptide caused permeability and disruption of the *F. graminearum* hyphae when applied as cell free antimicrobial. The growth

phase stages and condition must be carefully considered during the selection process when employing the *in vitro* culture methods. Biocontrol candidate strains secreting antimicrobial compounds have varying growth requirements that enhance the production of their active metabolites.

2.2.2 *In vitro* molecular approaches to detecting biocontrol strains and identifying the mechanisms they employ against phytopathogens

Detection of novel candidates harboring novel or known antimicrobial compounds and detection of novel antimicrobial compounds in known candidates is important. However, understanding the mode of actions of the antimicrobials secreted by these candidates cannot be underplayed. A combination of action mechanisms not limited to antibiotic secretion, ethylene production regulation, iron sequestration and nutrient competition, hydrogen cyanide production, solubilization of phosphate, release of lytic enzymes, hormone phytostimulation, acetoin and butanediol production are employed by biocontrol organisms to suppress plant pathogens. Mechanism of actions utilized by beneficial organisms have been adequately reviewed (Lugtenberg and Kamilova, 2009; Babalola, 2010; Raaijmakers and Mazzola, 2012; Ali et al. 2015).

To better understand plant-microbe interactions, novel and current techniques such as microarray systems, quantitative real time PCR (qRT-Pcr), subtractive hybridization, serial analysis of gene expression (SAGE), northern blotting and next generation sequencing (NGS), have been employed. These approaches have given better insights to gene expression, have helped select robust biocontrol candidates and also increased the knowledge of the capabilities possessed by well-known biocontrol candidates (Knief et al. 2014).

Using molecular genetics (mutant selection, transposon mutagenesis), genomics (PCR techniques), one can deliberately target candidate strains harboring two or more biosynthetic genes while discarding strains lacking the presence of known biosynthetic gene clusters (Mousa et al. 2015 and Shehata et al. 2016b). Metagenomics studies evaluating microbial diversity of resident flora of symptomatic/diseased crop/plant parts (stalks, stems and roots), rhizosphere soil, endosphere and phyllosphere for suppressiveness have been employed to run a comparison between low fusarium colonized samples and high colonized ones (Kohl et al. 2015).

Genomic and structural analysis are valuable for the unraveling of the functional assets of beneficial organisms. A transposon mutagenesis and whole genome mining of *Paenibacillus polymyxa* strain A26, revealed that the *P. polymyxa* mode action against *F. graminearum* and *F. culmorum* was not limited to production of fusaricidin but also involved polymyxins and other novel non-ribosomal lipopeptides (Abd El Daim et al. 2015).

2.2.3 Greenhouse evaluation of promising biocontrol strains

Following *in vitro* assessments, most research proceeds to conduct greenhouse experiments to ascertain the efficacy of selected microbial antagonists applied to crop seeds for protection against pathogen infection. The greenhouse controlled assay provides a promising strain of BCA with a stable environmental condition for survival and proliferation. Here, commercial potting soil or synthetic mixes of loamy sand, sandy clay loam or sandy loam soil may be used as soil medium. The soil could be pasteurized or heat-treated to create an axenic or gnotobiotic condition. Also, light intensity, water availability, soil amount, seed type and pot size are controlled. Greenhouse experiments are employed to evaluate any

potential BCAs that passed *in vitro* test. Results obtained from the greenhouse show more reliability and are most consistent to produce a BCA that passes on to commercialization. Some potential isolates from the *in vitro* studies become less effective or very ineffective during the greenhouse experiments. *Bacillus mojavensis* strain RRC 101 a patented endophyte for the biocontrol of seedling blight of wheat caused by species within the *F. graminearum* complex, was antagonistic *in vitro* against *F. graminearum* and seven other related species but was ineffective against *F. pseudograminearum* and *F. verticillioides* during growth room assay (Bacon and Hinton 2007).

Fifty-two bacterial strains and six *Trichoderma* spp. isolated from the wheat rhizosphere by Dal Bello et al. (2002), were evaluated for the management of *F. graminearum* seedling blight. The experiments conducted both *in vitro* and in the greenhouse showed that the isolates varied in their ability to inhibit the mycelial growth of *F. graminearum* in agar plate test. During the greenhouse assay, all isolates evaluated for disease reduction exhibited a pronounced suppressive effect. However, among the isolates, *Stenotrophomonas maltophilia* was the most effective of the antagonists in the growth chamber experiment, showing the best efficacy in suppressing damping-off. In addition, its performance was more consistent compared with other isolates during the experiment upon observing the wheat dry weight.

The ability of two strains of *Pseudomonas corrugata* to control disease manifestation in maize by *Pythium ultimum*, *P. arrhenomanes* and *F. graminearum* phytopathogens was evaluated by Pandey et al. (2001). Petri dish assays and plant based bioassay under glasshouse conditions were used to determine the suitability of the *Pseudomonas* isolates as biocontrol agents. The two strains suppressed the three maize pathogens and improved plant

growth when compared to the untreated plants infested with pathogen. The strains were also found to exhibit high antibiotics resistance, a potential that could enhance their environmental survival rate.

He et al. (2009) sorted for *F. graminearum* antagonist from soil and food isolate sources. Two strains of *Paenibacillus polymyxa* strain C1-8-b and W1-14-3, were identified as best candidates for inhibiting *F. graminearum* and reducing the production of the mycotoxin (DON) in the screen house. Their research utilized five assays that proceeded from a co culture, dual culture assay, an indirect impedance assay, a wheat floret assay, and ended with an assessment of DON production in the greenhouse.

Moussa et al. (2013), evaluated the efficacy of 18 selected bacterial isolates against the wheat phytopathogen *F. graminearum*. Two of the highly antagonistic isolates identified as *B. subtilis* strain MAA03 and *Pseudomonas fluorescens* strain MAA10 showed strong *in vitro* inhibition of the pathogen and were further used for treatments in the greenhouse experiment. The use of the strains either in combination or separately, positively affected the growth parameters evaluated. The type of treatment applied affected the growth of the crop and development of leafs. Increases in the root length, root fresh and dry weights and the number of leaves were recorded. Seed coating and soaking treatment showed significant positive results while soil drenched treatment showed negative results, as the shoot length decreased significantly.

Pal et al. (2001), showed that *P. fluorescens* strain EM85 and two strains of *Bacillus* MR-11(2) and MRF, from maize rhizosphere, antagonized *F. moniliforme, Macrophomina phaseolina* and *F. graminearum* using both *in vitro* and greenhouse single and co-inoculation

assays. The antifungal metabolites detected in the fluorescent *Pseudomonas* sp. EM85, *Bacillus* sp. MR-11(2), and *Bacillus* sp. MRF were also extracted and purified by thin layer chromatography bioassays and were found to inhibit the growth of *R. solani, F. graminearum, M. phaseolina, F. moniliforme* and *F. solani* strongly *in vitro*. The organisms proliferated and colonized the root zones aggressively.

Two greenhouse trials conducted by Mousa et al. (2015), during summers of 2012 and 2013, showed that an endophytic specie of *Citrobacter* and three endophytic strains of *Paenibacillus polymyxa* strain (4 isolates from 215 endophytic bacteria screened) suppressed the manifestation of *F. graminearum* infection and symptoms in maize. The four isolates were the most potent of the 215 isolated from three varieties of maize (wild, traditional and modern maize) exhibiting broad antifungal spectrum during *in vitro* analysis. The three *P. polymyxa* strains (1D6, 4G12, and 4G4) were isolated from the wild maize and harbored the biosynthetic gene responsible for the synthesis of fusaricidin an anti-fusarium compound. The *Citrobacter* strain (3H9) from modern maize, was the least potent among the four, it however caused higher DON reduction during storage.

2.2.4 Field trials conducted with potential biocontrol strains

Under field trials, fluctuations in climatic conditions are naturally expected and it is significant to analyze how environmental factors will affect the biocontrol effectiveness and survival of the BCA's. Field trials under pristine conditions helps in selecting BCA's that will be utilized in further studies. The viability and biocontrol capacity of several antagonists reduce when applied in the field compared to the greenhouse assays. During the field trials, the effectiveness of BCA in suppressing disease severity and toxin production, its ability to tolerate environmental conditions and remain viable both during field application and in

storage is crucial. Few articles have reported the progression from *in vitro* tests directly to the field experiment. The premise is that bypassing the greenhouse to field trials gives a true indication of the degree of suppression likely to be exhibited by a potential biocontrol strain in real time environmental situation.

Lysobacter enzymogenes strain C3 was identified as a bacterial antagonist of *F. graminearum* by Jochum et al. (2006). After establishing the effectiveness of the bacterium in suppressing the pathogenic effects of *F. graminearum* and improving the growth of several wheat cultivars through induced resistance in the greenhouse, they further carried out four field experiments to ascertain the suitability of the strains as a biocontrol agent for FHB. When used singularly as crop treatment, the bacterium showed inconsistencies in the field. However, its potency was restored when combined with the fungicide tebuconazole.

Similarly, Luongo et al. (2005) studied the efficacy of several saprophytic fungi isolated from necrotic plant tissues, stubbles, straw, seed surfaces, phyllosphere or roots of cereal crops to suppress the sporulation and saprophytic colonization of several pathogenic *Fusarium* spp. on wheat and maize crop residues and *F. graminearum* was part of the toxigenic fusarium tested against. Bioassays carried out include, *in vitro* petri dish test and preliminary field test that involved applying several of the antagonists to pieces of maize stalks and flowering maize ears. From the saprophytic fungi, the antagonist most effective against *F. graminearum* was *T. harzianum*. *Clonostachys rosea* exhibited the strongest inhibition against all the six *Fusarium* spp. tested by reducing their sporulation. The other antagonists that showed appreciable inhibition against *F. proliferatum* and *F. verticillioides* were *C. cladosporioides* and *F. equiseti* and *Trichoderma viride*. They however failed to

inhibit *F. culmorum* or *F. graminearum*. It is suspected that the *Clonostachys rosea* strains suppressed by competitive colonization.

Bacillus SG6 effectively inhibits both growth and sporulation of *F. graminearum in vitro* (Zhao et al. 2014) and its effectiveness is more pronounced than that of carbendazim, the chemical fungicide widely used in China for the control of FHB. Application of the strain caused significant changes in several parameters considered (such as crop yield, FHB index and DON production) during the field trials. Ultrastructure observation of *F. graminearum* treated with SG6 using the Scanning electron microscope (SEM) and transmission electron microscope showed disruption of fungal hyphae and cell wall lysis. Strain SG6 harbors five genes (*bmyB, fenD, ituC, srfAA and bacA*) known for the secretion of antimicrobial peptides. Also, Khan et al. (2004) investigated the ability of three *B. subtilis* strains, one yeast strain and three *Cryptococcus* strains to antagonize *F. graminearum* in wheat field trials. These antagonists (*Cryptococcus* sp. OH 181.1, *Cryptococcus sp.* OH 71.4, and *C. nodaensis* OH182.9) tested in the greenhouse exhibited geographical stability.

2.3 Efficacy and stability of biocontrol strains

Regarding the survivability and effectiveness of a BCA in the field, producers and end users of the BCA's give different report. End users complain that BCA's are inconsistent under natural conditions compared to the expected information the scientist introducing the BCA gives. Because the scientist has proved the effectiveness of the BCA in the experimental tests, he has high reliability on the product. However, most BCA's that have been proven stable and effective had to pass a series of evaluative tests by the end users even after the field trials carried out by the scientist. The scientists must therefore carry out their experimental test to suit the needs of expected end users. During trials, scientists must put

into consideration critical factors that could influence the effectiveness and stability of the BCA. Such factors include the soil in which the BCA would be introduced, genotype of plant for which the BCA is being manufactured, delivery mode by which the BCA would be applied, the beneficial, neutral or detrimental effects the BCA would have on its host environment (Kamilova and Bruyne 2013). The durability of a BCA is measured by its degradation or loss of effectiveness during field or on the farm applications. Geographical instability has been recognized to be responsible for the ineffectiveness and inconsistences of several commercial biocontrol strains outside their indigenous environment (Ahmad et al. 2008; Abiala et al. 2015; Bardin et al. 2015).

2.4 Modes and conditions of application of candidate biocontrol during experiments

Treatment types, inoculum dosage, formulation types, vehicle of delivery and storage of microbial inoculants/formulations and application times have a significant effect on the effectiveness of a candidate BCA designed for crop protection. A major limitation identified for BCA's is the continuing environmental fluctuations they encounter *in planta* and the challenges encountered in developing a stable product formulation. The copyright issues protecting and guiding production industries also make it difficult to get information regarding the recipe for commercialized microbial formulations. All this must considered when designing field trials for crop plants. Few of the utilized vehicles of delivery and storage in recent times include liquid and mineral carriers, protectants, organic carriers, desiccants, stabilizers, UV protectants, binders and stabilizers (Schisler et al. 1997). Palazzini et al. (2016a) utilized physiologically improved cells (vegetative cells) and also a spore treatment for the field trial they conducted with *B. subtilis* RC 218. Dry powder formulations when

used to deliver microbial formulations and freeze dried formulations when used in spray-dryers for BCA delivery reduce production costs and increase processing rates.

2.5 Semicontrolled experimental conditions

Most of the studies described in this review utilized sterilized soils in their greenhouse assays. This approach is necessary and suitable because it helps detect and compare the effectiveness of the BCA in the treated plants and untreated plants under the identifiable external factors introduced. It also requires a shorter time and one experimental location to determine the results of the assay. In contrast however, field trials which involve the use of natural unsterilized soils during experimental periods to assess the relationship between the plant, the phytopathogen, environmental factors (biotic and abiotic) and the biocontrol strain being introduced are quite expensive and require a minimum of two years and two experimental sites (Vacheron et al. 2016). In as much as these field trials are necessary for predicting the real time efficiency of the potential biocontrollers, another approach to circumvent the need for the expensive field trials might be the use of unsterilized soils in semicontrolled trials.

In semicontrolled trials, soils are taken from the locality from which field trials ought to be carried out and used also in greenhouse pot experiments under natural atmospheric conditions such as open rainfed conditions and exposure to direct sunlight (Mao et al. 1998 and Mehnaz et al. 2010). The semicontrolled trial would be a combination of several aspects of both the greenhouse test and field trials. The assay should provide a closer picture to what happens in the field, compared to the sterilized soils utilized in controlled greenhouse pot assays and the closely monitored growth conditions employed. In the semi-controlled experiments the premise is that, the potential biocontrollers will be proliferating along with

both the indigenous microbes present in the soil and the introduced phytopathogens. Though the quantity of soil is controlled, the quality is not controlled. Furthermore, the quantity and concentration of BCA and phytopathogen inoculum being introduced during the experiment still remains controlled (Abiala et al. 2015)

Soil biophysiological parameters and management practices have been shown to have diverse effects on soil microbial community (Akhtar et al. 2000 and Babalola 2010) and soil type remains a major determinant of community structure for the microbial communities. Furthermore, bacterial inoculants in unsterilized soil stimulated better growth effects in maize compared to sterilized soil (Singh et al. 2007 and Johnston-Monje et al. 2014). Soil amendment must be considered when designing biocontrol studies, because it plays a significant impact in the efficacy and survivability of BCAs.

A key requirement of a biocontrol strain targeted for use against soil borne phytopathogen is its competence in the rhizosphere. It must compete adequately with indigenous microbial populations within the environment of the rhizosphere and colonize the root surface (Ambrosini et al. 2015 and Khabbaz et al. 2015). The use of untreated soil could also help ascertain the survivability of the potential strain and its effect on non-target indigenous microbial populations present in the soil. Therefore, utilizing an untreated soil for a semicontrolled field trial would be more suitable because most traditional farmers and large-scale maize producers are reluctant to introduce phytopathogens into their field with the promise that a novel biocontrol product will eradicate the outbreak or emergence of disease that could ensue. Once the competence is proven in the semicontrolled field trial thus bypassing the several expensive preliminary field trials, the biocontrol strain can then be introduced for further trials.

Another aspect of the semicontrolled experimental conditions that would reduce cost and time of selecting a candidate biocontrol strain for field application is the use of established strains having plant growth promoting traits (figure 2). In recent times, more authors are conducting their *in vitro* analysis, greenhouse experiments and field trials with microbial strains previously isolated by other researchers. This enables them to bypass the laborious stage of sampling, isolation and identification of potential isolates and determination of mechanism of action of such isolate (Dunlap et al. 2011 and Grosu et al. 2015).

Despite this reviewed list of potential biocontrollers from different microbial phyla and genera (table 2), many of these bacterial control agents are yet to be adopted for commercial use. This could be attributed to the geographical diversity found in the *F. graminearum* species and the geographical instability of its potential biocontrollers (Zhang et al. 2012).

Table 2.1: Studies on maize involving biological control agents and *F. graminearum*

Utilized Plant Growth Promoting Microorganism	Source	Function	Conditions of Study	Mode of Activity	Reference
B. subtilis (SG6)	Anthers of luffa	Antagonist for *F. graminearum*	IVT and FT	Enzyme secretion (Chitinase) & Lipopeptide production (fengycin and surfactin)	Zhao et al., (2014)
B. subtilis (D1/2-DAOM 231163)	Cultivated soil	Antagonist for *F. graminearum*	IVT, GHE and FT	Lipopeptide production (fengycin)	Chan et al., (2009)
Acremonium zeae	Maize endosphere	Antagonist for *F. graminearum* and *F. verticillioides*	IVT	Lipopeptide production (pyrrocidines A and B)	Wicklow and Poling (2009)
Paenibacillus polymyxa and *Citrobacter* sp.	Wild maize (teosinte) endosphere	Antagonist for *F. graminearum*	IVT and GHE	Lipopeptide production (fusaricidin)	Mousa et al., (2015)
B. subtilis (BS-918)	Coastline soil	Antagonist for *F. graminearum*	IVT	Lipopeptide production (fengycin A & B)	Chen et al., (2014)
Lipopeptide extract of *B.*	Deep-sea sediment	Antagonist for *F. oxysporum*	IVT	Lipopeptide production (fengycin A &	Chen et al., (2010)

amyloliquefaciens (SH-B10)		and *F. graminearum*		6-Abu fengycin)	
Pseudomonas spp. (DGR22, MGR4 and MGR39)	Maize field rhizosphere, endorhizosphere and bulk soil	Antagonist for *F. verticillioides* RC2000, *F. solani*, *F. graminearum* RC 664, *F. proliferatum* RC 479,	IVT	Secretion of enzyme (protease), siderophore production, lipopeptide secretion (pyrrolnitrin) & HCN production	Cordero et al., (2012)
Bacillus methylotrophicus	Corn plants	Antagonist for *F. graminearum*	IVT and FT	Unidentified	Li et al., (2016)
Paenibacillus sp. (5 L8)	Maize endosphere	Antagonist for *F. graminearum*	IVT	Enzyme secretion (β-1,3-1,4-glucanase)	Liu et al., (2015)
Essential oils	1. Rocket seeds (*Eruca sativa*), 2. Rosemary (*Rosmarinus officinalis*) and 3. Tea tree (*Melaleuca alternifolia*)	Suppression of *F. avenaceum*, *F. moniliforme*, *F. semitectum*, *F. solani*, *F. oxysporum*, and *F. graminearum.*	IVT	Unclarified volatile & non-volatile components (1. isothiocyanate compounds, glucoerucin & other flavonoids 2. monoterpens ápinene, 3. terpinen-4-ol,	Sahab et al., (2014)

				γ-terpinene & α-terpinenen	
Trichoderma virens, Burkholderia cepacia strains Bc-B and Bc-1	Culture center, commercial seed, maize rhizosphere	Antagonist for *F. graminearum*	GHE	Unspecified	Mao et al., (1998)
Pseudomonas spp.	Maize rhizosphere	Defense response against *Fusarium* sp.	IVT	Phytohormone production (indole-3-acetic acid) & carbon utilization	Lawongsa et al., (2008)

Abbreviations: GHE = Greenhouse experiment; IVT = *In vitro* test; FT = Field trials, N_2 = Nitrogen, IAA = Indole-3-Acetic Acid, ISR/SAR = Induced systemic resistance/Systemic acquired resistance

2.6 Monitoring and ensuring effectiveness of BCAs

Lack of effectiveness and reduced effectiveness of BCA formulations during field trials and commercialization have been reported and these inconsistencies have often been attributed to several factors such as variations in climatic conditions, innate potentials of pathogen, instability of BCA during storage and application (Ruocco et al. 2011 and Bardin et al.,2015). Velivelli et al. (2014) and Varga et al. (2015), suggested that to ensure that the performance of a potential biocontroller that has undergone *in vitro*, greenhouse and controlled field trials is consistent, such a biocontroller should be tested in multi-geographical sites, under different climates, against vast a range of pathogens and diverse crops. The potential BCA's should be isolated from the soil environment in which they will be used

(Howell et al. 2003 and Small et al. 2012). However, the economic and financial implications of such project will be enormous. Another challenge to monitoring effectiveness would be what to monitor and when should monitoring take place. Should control strategies targeting DON contamination, disease severity, reduction in pathogen population and mycotoxin be pre-harvest or postharvest?

The high cost of carrying out *in planta* studies, the unwillingness of the end users or farmers to try out something new, and the difficulty in getting funds or industrial partners are major challenges encountered prior to the formulation of a potential microbial strain to a commercialized state. These are major reasons why most of the potential strains identified in various geographical zones during *in vitro* and greenhouse studies are yet to be used for *in planta* studies or formulated for commercialization (Ash 2010; Bailey et al. 2010; Kohl et al. 2011; Ruocco et al. 2011). Few reports on the control of DON contamination by BCA's are available probably because research focusing on such areas is still in developmental stages (Wegulo et al. 2015).

Since most potential antagonists are selected following the screening approaches described in **figure 1**, it is likely that a large number of microbial isolates can be found showing antagonism in experimental studies yet they are not suitable for commercial use. A potential biocontrol strain may be too expensive for mass fermentation production; their inoculum during product formulation may not have a long shelf life and their target end users may not be many enough for their industrial implementation. A good screening approach would include commercial aspects in the early stage of selecting a potential biocontrol strain, ensuring the strains meet the specifications needed for commercial application (Kamilova and Bruyne 2013). Schisler and Slininger (1997) focused both on bioefficacy and growth

kinetics of BCA candidates during their feasibility studies for identifying potential commercial strains. They divided their screening process into three categories: (1) the necessity of choosing an appropriate pathosystem; (2) the importance of having an appropriate method for microbe isolation, and (3) the necessity of determining the appropriate isolate characterization and performance evaluation procedures.

Most biocontrollers that show broad-spectrum activity *in vitro* against phytopathogen have not been effective as broad spectrum *in planta*. A need for narrow spectrum biocontrollers targeted at specific strains might be a better approach for alleviating this challenge. Our review shows that the majority of studies affecting major cereal grains focused on alleviating fusariosis through a broad-spectrum antagonism approach (Table 2.2).

Table 2.2: Biocontrol studies on major cereal grains involving the phytopathogen *F. graminearum*

Utilized Plant Growth Promoting Microorganism	Source	Cereal studied for *F. graminearum* control	Conditions of Study	Mode of Activity	Reference
Pantoea sp. *Paenibacillus* spp., *Bacillus* sp. or *Fictibacillus* sp.	Wheat seeds	Wheat & Barley	IVT & GHE	IAA production, biofilm formation, phosphate solubilization	Herrera et al., (2016)
B. subtilis (D1/2)	Cultivated soil	Wheat	GHE	Lipopeptide production (fengycin)	Chan et al., (2009)
B. subtilis spp. (AS 43.3, AS 43.4 & Cryptococcus *flavescens* OH 182.9)	Wheat anthers	Wheat	IVT, GHE & Field	Lipopeptide production (iturin, fengycin, surfactin)	Khan et al., (2004)
B. amyloliquefaciens (S76-3)	Diseased wheat spikes	Wheat	IVT, GHE & FT	Lipopeptide production (iturin, surfactin, plipastatin)	Gong et al., (2015)
B. amyloliquefaciens (B3, BW and BIR), *Bacillus* spp. (B1, B5),	Compost tea & Soil	Wheat	IVT & GHE	Unidentified	Grosu et al., (2015)

Paenibacillus polymyxa (W1-14-3 & C1-8-b)	Concurrent screening of multiple soil & food	Wheat	IVT & GHE	Unidentified	He et al., (2009)
Pseudomonas chlororaphis subsp. *Aurantiaca* (Pcho10)	Wheat head	Wheat	IVT & GHE	Lipopeptide production (phenazine-1-carboxamide)	Hu et al., (2014)
Streptomyces sp. (BN1)	Rice kernels	Wheat	IVT & GHE	Unidentified	Jung et al., (2013)
B. methylotrophicus	Corn plants	Maize & Sorghum	IVT & FT	Unidentified	Li et al., (2016)
Burkholderia spp., *Streptomyces* spp., *Bacillus* spp., *Pseudomonas* spp., *Paenibacillus* spp., *Rhizobium* spp., *Dyella* sp., & *Mesorhizobium* spp.	Tillage & no-tillage soil	Black Oat & Cowpea	IVT	Secretion of volatile compounds & siderophore production	Lisboa et al., (2015)
B. megaterium (BM1) and *B. subtilis* (BS43, BSM0 & BSM2)	Maize endosphere	Wheat	IVT & FT	Unidentified	Pan et al., (2015)
B. amyloliquefaciens WPS4-1	Peanut shells	Wheat	IVT	Lipopeptide (iturin)	Shi et al., (2014)

B. methylotrophicus (BC79)	Primeval forest soil	Rice	IVT, GHE, FT	Phenaminomethyl-acetic acid	Shan et al., (2013)
Trichoderma gamsii (6085)	uncultivated soil	Wheat	IVT and FT	Unclarified (secretion of chitinase)	Sarrocco et al., (2013)
B. pumilus (SG2)	Saline desert	Wheat and Barley	IVT	Secretion of enzyme (chitinase)	Shali et al., (2010)
Clonostachys rosea strains (016)	Plant research international (Netherlands)	Wheat	FT	Competitive colonization	Palazzini et al., (2013a)
B. subtilis (RC 218) & *Brevibacillus sp.*(RC 263)	Wheat anthers	Wheat	FT & SCFT	Unclarified (antibiosis)	Palazzini et al., (2013b)
C. rosea, Cladosporium cladosporioides & Trichoderma spp.	Multiple locations	Wheat	IVT & FT	Competitive colonization	Schoneberg et al., (2015)

Abbreviations: GHE = Greenhouse experiment; IVT = *In vitro* test; FT = Field trials

Increased protection of wheat against phytopathogens has received more priority than other cereal grains. This perhaps is due to the broad-spectrum antagonistic approach employed whereby an antagonist must not only suppress its main target pathogen but must suppress other closely related species or genera. Formenti et al. (2012) confirmed the differences that exist in efficacy of broad spectrum fungicides against different fungal pathogens. Their research showed that the variability seen in the suppression of *F.*

verticillioides and *A. flavus* using the same fungicide was significant. The diverse strain found in the FGSC complex could make the broad application of an effective biocontrol strain in broad geographical zones almost impossible.

2.7 Current approaches to understanding plant-microbe interaction

In recent years, efficiently designed RNA interference (RNAi) based plant protection approaches that involve RNAi of important biosynthetic pathways in invading pathogens are being employed. The RNAi technique is a gene attenuation method used to develop transgenic pathogen resistant crops and identify gene function in microbes. These plants possess the RNAi machinery which they use as defense mechanism (RNA silencing or co-suppression mechanism) against invading pathogens (Schumann et al. 2013; Cairns et al. 2016; Wang et al. 2016). To silence an endogenous gene in a plant, an effective method will be to transform the plant with a gene construct that encodes a hairpin RNA (hpRNA) (Helliwell and Waterhouse, 2003). The constructs are expressed in plants through plasmid delivery, viral or bacterial vectors and target specifically the invading microbe they are supposed to silence (Wani et al. 2010). Currently, improved RNAi methods are being exploited in plant management strategies, some targeting the *Fusarium* spp. (Schumann et al. 2013; Chen et al. 2015; Koch et al. 2016).

The phenome based functional analysis approach has been employed recently for understanding functionality of candidate biocontrol agents targeted against maize fusariosis. It can also be used in the identification of putative transcription factors (TFs), characterization of functional genes and identification of unique traits in phytopathogens such as *F. graminearum* (Son et al. 2011).

Another breakthrough that has revolutionized the application of NGS is the use of RNA sequencing (RNA-seq) in transcriptome research. During RNA-seq, a transcriptome, being a collection of all the transcripts (RNAs) present in a given cell, is evaluated qualitatively and quantitatively at a particular moment of cell development or during a specific physiological condition (Wang et al. 2009), yielding unbiased transcripts with dynamic detection range (Marguerat and Bahler 2010 and Pinto et al. 2011). RNA-seq analysis can be used for both exploratory and quantitative assessment, from understanding the transcriptional profile of biocontrol agent (identification of genes involved in biofilm formation and secretion of secondary metabolites) to understanding the resistance elicited by crops against phytopathogens. Activation of antibiotic genes in BCA and defense related genes in maize have been identified to be time dependent after infection of maize by *F. graminearum* using RNA-seq (Zhang et al. 2015 and Krober et al. 2016). Furthermore, RNA-seq has been used to determine the mechanisms that regulate microbial metabolite synthesis (either in pathogen or BCA) and metabolite function in the microbial cell, which has become a major priority for plant pathologists (Sieber et al. 2014 and Mwita et al. 2016).

2.8 Perspectives

The approaches and technologies mentioned above obviously come with their merits and demerits. However, a combination of these approaches would ensure rapid selection of biocontrol candidates and further enhance current plant disease management strategies that will help reduce fusariosis and increase maize yield. Most of the novel approaches to understanding host-pathogen relationships in plants are now geared towards identifying plant-mediated responses to pathogen or BCAs such as induced systemic resistance (ISR). However, few reports are available on transcriptomics studies involving cereals, fusarium

and rhizobacteria. The combination of the technologies will allow the identification of the functional genes in both the host plant and the colonizing microbe (pathogen or BCA). Where the attenuation or amplification of the identified functional gene is required (either in host or colonizer), such can be readily implemented.

Because of the growing need for a competent biocontrol strain in field trials and the continued reservations for genetically modified BCAs in some parts of the world, culture dependent selection methods for screening candidate strains will still be widely employed. However, rather than every laboratory in the field searching for its own highly effective biocontroller, efforts should be garnered at making already identified indigenous laboratory candidate strains or academically promising strains developed, commercialized and adopted in the market. An alternative approach for screening and developing a biofungicide for commercialization could be by passing several of the laborious stages in identifying and selecting a potential biocontrol strain (figure 2.2).

A collaborative effort of diverse scientific disciplines appears to be the future for the biofungicide industry. Right from the start to the finish, the manufacturing companies and industrial world should become involved in the process of selecting the correct biological control agent through to the selection of a commercial brand. The collaboration should not be lacking in funds during the process. The funds and infrastructures should be sourced or provided by various entities and institutions.

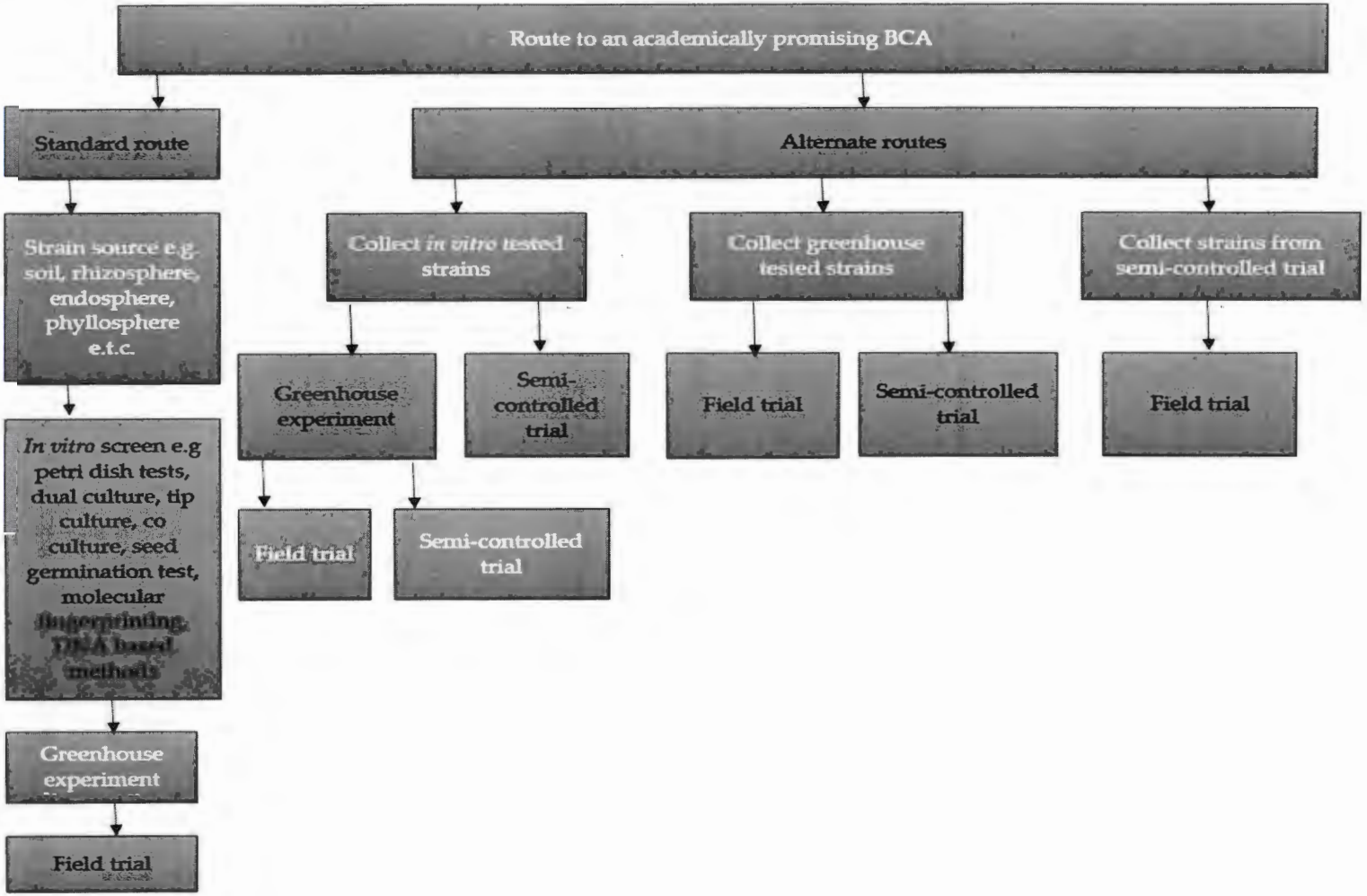


Figure 2.2: Route to academically promising biocontroller

2.9 Concluding remarks

In this chapter, we have investigated several means of screening for potential biological agents that could be used to control *F. graminearum* fusariosis in maize. We are of the opinion that a narrow spectrum biofungicide might be the most effective in controlling the continued emergent, rapid spread of fusariosis in maize and so there will be need for further studies that concentrate solely on pathogen specificity of a BCA. There are still drawbacks in the field application and commercialization of currently available potential BCAs that have passed greenhouse trials, therefore this area should be pursued.

CHAPTER THREE

SCREENING FOR LIPOPEPTIDE PRODUCING, *FUSARIUM GRAMINEARUM* SUPPRESSING *BACILLUS* SPP. FROM MAIZE RHIZOSPHERE

Abstract

The versatile genomic potential of the *Bacillus* spp. ensures the continued relevance of this well studied Gram-positive spore forming bacteria in biological plant disease management strategies. We sought to provide an indigenous *Bacillus* strain with antagonistic ability against the maize phytopathogen *Fusarium graminearum*. Using *in vitro* chromogenic and molecular methods we identified several *Bacillus* isolates with varying inhibitory capacity against the two fungal pathogens (*F. graminearum* and *F. culmorum*) known to cause fusariosis in small cereal grains. Seven isolates with strong inhibitory capacity were phylogenetically characterized using 16S rRNA gene after which PCR amplification of encoding genes using specific primers sets, identified the lipopeptides iturin, surfactin and fengycin to be responsible for the antifungal activities of some of the isolates. The biosuppressive effects against phytopathogens were strongest and most consistent for isolates NWBS10.5, MOLBS8.5, VERBS5.5, MOREBS6.3, MARBS2.7, MORWBS1.1, and MOLBS8.6. A few of the isolates showed possible biosurfactant capability and their susceptibility to known antibiotics is indicative of their environmental friendliness.

Keywords: antifungal, lipopeptides, *Fusarium*, *Bacillus*, rhizosphere

3.1 Introduction

In recent years, focus has shifted from the use of environmentally harmful plant disease management practices such as the use of chemical pesticides and inorganic fertilizers to the development of ecologically friendly approaches that do not pose a public health threat. The use of biological control agents (BCAs) in the management of cereal crop diseases have become popular (Lugtenberg and Kamilova 2009; Etebarian and Glicks 2012; Dimkic *et al.*, 2013) and bacteria species from the genus *Bacillus* have gained prominent attention due to their tolerance to high temperature, ability to withstand adverse environmental conditions, ability to grow rapidly in liquid mediums and their ability to produce a large variety of secondary metabolites that have broad antimicrobial capabilities (Dunlap et al., 2011; Santoyo et al., 2012; Sumi et al., 2014).

Members of the Gram-positive endospore forming *Bacillus* spp. such as *B. amyloliquefaciens* and *B. subtilis* collected from plant parts have been used in the control of fusariosis in small cereal grains including maize (Bacon and Hinton, 2011; Gond et al., 2015). Fusariosis in maize, which could manifest as Fusarium head blight (FHB) or Fusarium ear rot (FER) and many more, is caused by members of the fusarium such as *Fusarium graminearum* and *F. verticillioides* and these diseases affect maize production in South Africa and other areas of the world (Summerell et al., 2011; Boutigny et al., 2012). Maize is a staple crop in South Africa consumed daily in most households and used in the production of animal feeds (Lamprecht and Tewoldemedhin 2011; Janse van Rensburg et al., 2015), therefore efforts to reduce losses due to pre-harvest and post-harvest contamination by *F. graminearum* infection have recently gained significant attention (Boutigny et al., 2011; Mngqawa et al., 2016). The presence of mycotoxins zearalenone and deoxynivalenol found in maize grains infected by *F. graminearum* is also cause for concern (Wang et al., 2011).

With the reports that BCAs used for crop protection perform better in their native geographical regions due to increased survival rate compared to the use of imported commercial BCAs (Pereira et al., 2010; Grzywacz et al., 2014; Abiala et al., 2015; Bardin et al., 2015), our goal in this present work is to collect indigenous *Bacillus* strains from the maize rhizosphere, evaluate their anti-phytopathogenic potentials *in vitro* against *F. graminearum*, molecularly characterize the *Bacillus* isolates and identify the likely mechanisms they employ in their anti-phytopathogenic activities. *Bacillus* spp. secrete lipopeptides compounds such as surfactin, fengycin and iturin which they utilize in antibiosis. The presence of these cyclic lipopeptides in our maize root associated strains would be of value if they are to be considered as potential biocontrol agents for the management of *F. graminearum* infections.

3.2 Materials and Methods

3.2.1 Sampling location and geographical description of sampling sites

Rhizosphere soil samples were collected randomly in no particular order from ten maize farms at harvest time. Geographic location of the sampling sites covers 28,206km^2 area. Temperature ranges between 17°C-31°C during the summer and between 3°C-21°C during the winter with average rainfall of 360 mm.

3.2.2 Sample collection from rhizosphere

Depending on the width of the maize plot and in no particular order, 20 -30 grams rhizospheric soil were sampled randomly from four maize rows 15 m-25 m apart in each plot from the ten maize farms in the North West Province of South Africa. Harvested maize plants were shaken gently at the roots to manually remove the loosely attached soil. The adhering root soil was considered as the rhizosphere soil and these were pooled for each location. We obtained a total of 10 different soil samples from the different maize plots.

3.2.3 Differential and selective and isolation of Bacillus spp. from rhizosphere sample

Five grams of each soil sample was inoculated in 45 ml of LB broth and incubated for 16 hr with continuous shaking at 150 rpm in an incubator at 35°C after which a calibrated inoculating loop was used to streak on the surface of 20 HiCrome™ *Bacillus* commercial Agar plates without polymyxin supplement and 10 *Bacillus* agar with polymyxin supplement. HiCrome *Bacillus* agar is used for rapid identification of *Bacillus* spp. from a mixed culture by chromogenic method. Following manufacturer's directions, distinct colonies were randomly selected from the agar plates of each sample (Table 3.1).

3.3.1 Gram staining, oxidase tests and catalase activity

To presumptively ascertain the genera of the selected isolates, Gram staining was performed using a Gram-stain kit (HiMedia). Oxidase activity was determined using 1% (w/v) N, N, N', N'-tetramethyl-1, 4-phenylenediamine dihydrochloride. Catalase activity was confirmed by adding 3% (v/v) hydrogen peroxide solution to colonies grown on LB agar. Two hundred isolates were selected and maintained at −80°C in Luria–Bertani (LB) broth with 15% glycerol (v/v) and a 15 ml LB broth or agar slant of each isolate was kept at 4°C as working culture.

3.2.4. *In vitro* screening for *Fusarium* suppressing *Bacillus* isolates

3.2.4.1 Preliminary antagonistic activity

Fusarium graminearum and *Fusarium culmorum* were kindly provided by Dr Claire Prigent Combaret (UMR CNRS 5557) Microbial Ecology of Lyon. University Lyon 1, France and Prof Cristina Cruz CE3C, Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências da Universidade de Lisboa, Portugal respectively and they were maintained on Potato Dextrose Agar (PDA) plate.

Preliminary detection of the antagonistic activities of the 200 *Bacillus* isolates against *F. graminearum* was carried out by multiple confrontation dual culture tests. The protocols of Chen et al. (2014) were slightly modified. A 5 mm diameter plug from an actively a growing (7-day-old) mycelial culture of *F. graminearum* was placed in the center of freshly prepared PDA plates (90 mm). From the 200 *Bacillus* isolates initially selected, six fresh colonies from 24 hr LB agar culture were circularly streaked (equidistance 1.5 cm) along each PDA plate at a distance of 1.5 cm from the edge of the plate using a sterile inoculating loop. Control plates consisted of *F. graminearum* placed on PDA alone. The plates were further incubated at 28°C for 7 days. Thereafter, only 11 isolates (BS6.3, BS8.5, BS10.5, BS8.6, BS1.1, BS5.5, BS3.5, BS2.7, BS6.2, BS4.6 and BS4.3) exhibiting strong inhibition were selected for further antifungal confirmatory tests.

3.2.4.2 Confirmatory *in vitro* antifungal test

Plates were prepared as described above, however antagonism was carried out against two fungal pathogens (*F. graminearum* and *F. culmorum*) in three conditions. (a) Single loop full of bacterial antagonist streaked at the center of PDA three days before both fungal agar plugs were inoculated on opposite sides (condition 1); (b) Single loop full of bacterial antagonist streaked at center of plate while simultaneously inoculating both fungal agar plugs on opposite sides (condition 2); (c) Single loop full of bacterial antagonist streaked three days after the fungal agar plugs were inoculated on opposite sides (condition 3). The pseudomonads with strong inhibition zones around their streaks against the two pathogens in the screening plates were selected for further characterization. The antagonistic effect was determined by measuring the zones of inhibition (mm). The percentage of growth inhibition was calculated using the formula

$$PGI = [(C1 - C2)/C1] \% \ 100$$

where PGI is the percentage of growth inhibition, C1 is the control mycelia area of uninhibited fungi, and C2 is the distance of the bacterial colony to the growing edge of the fungal mycelia. Experiments were repeated three times and the values were recorded as the means of three replicates.

3.2.5 Susceptibility of antagonist isolates to antibiotics

Sensitivity of the selected isolates to the following antibiotics concentrations Pg-Penicillin (10µg/disc); Cip-Ciprofloxacin (5µg/disc); Rp-Rifampicin (5µg/disc); K-Kanamycin (15µg/disc); E-Erythromycin (15µg/disc); Gm-Gentamycin (10µg/disc); S-Streptomycin (25µg/disc); C-Chloramphenicol (25µg/disc); and Nor-Norfloxacin (5µg/disc) was done according to Khalifa et al., (2016). An overnight-old culture of each isolate was homogeneously spread on the Muller Hinton (MH) agar plates and the antibiotic discs were aseptically placed on the plates. Plates were checked for inhibition zones around the antibiotic discs after 48 hr incubation at 30°C. Strains sensitive to the antibiotics tested at concentration indicated showed a clearing zone. The actual diameter of the zone of inhibition was calculated by subtracting the diameter of the disc from the total diameter (Khalifa et al., 2016) and this was recorded for statistical analysis. The experiment was done in triplicate.

3.2.6 Detection of biosurfactant ability

3.2.6.1 Hemolysis blood agar test

Isolates (BS10.5, BS6.3, BS5.5, BS4.6, BS8.5, BS8.6 and BS1.1) were subjected first to hemolysis test as isolates with biosurfactant producing capability can lyse erythrocytes. A colony loopful from fresh cultures of each isolate or 20 µl of each fresh culture in LB broth was taken and streaked on Blood Agar plates (HiMedia, India). Plates were incubated from 48-72 hr at 37°C (Chakraborty et al., 2014). The plates were then observed for clear zones around the colonies.

However, a clearing zone around the bacterial colony on blood agar is not always confirmatory for biosurfactant production (Youssef et al. 2004; Hazra et al. 2011). We further conducted other confirmatory tests.

3.2.6.2 Drop collapse test and Microplate assay

To determine the production of biosurfactant compounds, a modified ''drop collapse test'' applied according to Yanes et al. (2012) was conducted. Briefly, each well of a 96 well plate lid was coated with 2 µl of test substances consisting of vegetable oil, motor engine oil, kerosene, hexadecane, parafilm and equilibrated for 2 h. A cell free supernatant from an overnight LB broth culture of each isolate was prepared by filtering through 0.22 µm and a 5 µl drop of the cell-free supernatant of each isolate was then placed in the center of the coated well. The result was determined visually after 1 min. If the drop remained beaded the result was scored as negative, and if the drop collapsed, the result was scored as positive. Water and SDS were used as negative controls of the media. Each treatment was repeated three times.

Drop collapse test and microplate assay were also carried out according to the method described by Belgacem et al. (2015). Briefly, 20 µl of the supernatant was added to the hydrophobic surface of parafilm and the shape of the droplet was inspected after 1 min. If the drop remained beaded, the drop assay was scored negative and if the drop collapsed, the result was positive. For the microplate assay 100 µl supernatant was pipetted into a well of 96-microplate and the plate was viewed using a backing sheet of white paper with black grid. A positive result is indicated by the surfactant causing some wetting at the edge of the well and the fluid taking the shape of a diverging lens. The negative result is based on the premise that pure water in a hydrophobic well shows a flat surface or an optical distortion observed when surfactants are added to an aqueous solution.

3.2.7 Extraction of genomic DNA

We further chose 7 isolates (BS10.5, BS6.3, BS5.5, BS4.6, BS8.5, BS8.6 and BS1.1) out of the 11 *Bacillus* isolates based on their strong antifungal activity for molecular analysis. Genomic DNA was extracted from overnight culture of the selected isolates using the Zymo Research ZR Soil Microbe DNA Miniprep genomic isolation kit (Epigenetics) following the manufacturer's procedure. DNA quantity and quality was assessed with spectroscopic methods using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and the DNA was used as the template for polymerase chain reaction (PCR) analysis.

3.2.8 Detection of lipopeptide genes and molecular characterization of *Bacillus* isolates

Identification of the selected *Bacillus* isolates was by 16S rRNA gene sequencing (Garbeva et al., 2003) and the presence of lipopeptide genes in the DNA extracts of the *Bacillus* isolates was determined with a 25 µl reaction mixture containing 1.5-2.5 µg of template DNA; 1 µl of primer, 12.5 µl OneTaq quick load 2X master mix with standard buffer (NewEnglandBiolabs NEB), 9.5-10.5 µl nuclease free water in PCR thermocycler. All the primers utilized in PCR amplifications were synthesized by Whitehead Scientific, Integrated DNA Technologies, and the targeted genes with during the PCR conditions are shown in Table 3.1. The PCR amplicons were analyzed by electrophoresis in 1% (w/v) agarose gel and the sizes of the bands were determined using 1 kb molecular marker. The gel containing ethidium bromide (10 µg/ml) were visualized and photographed using a gel documentation system (Gel Doc 2000, Bio-Rad) to confirm the expected size of the PCR products.

3.2.9 Sequencing reaction

Both the forward and reverse primers were used in the sequencing of the purified PCR products. The sequencing was done at Inqaba Biotechnical industrial (Pty) Ltd, Pretoria, South

Africa with PRISM TM Ready Reaction Dye Terminator Cycle Sequencing Kit using dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Bio systems, USA) by following the manufacturer's instructions. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analyzed using CLC Main Workbench 7.

16S rRNA sequences were blast searched on the NCBI GenBank and ENA database. Aligned sequences were analyzed using MEGA 7.0 software (Tamura et al., 2011) and phylogenetic trees were reconstructed based on the 16S rRNA gene using the neighbour-joining methods (Saitou and Nei, 1987). Topological robustness was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates.

3.3 Statistical analysis

A Multivariate General linear model was used to analyze treatment means, and inhibition rates. Least significant difference test (LSD), Duncan multiple test and Student-Newman-Keuls (SNK) test were used to compare observed means, pathogen-antagonist relationship, treatment effects and effect of conditions of inoculation using SPSS statistical package programme (version 22) at the significance level of 5%.

3.4 Results and Discussion

Table 3.1: Geographic sites and numbers of *Bacillus* isolates selected from samples collected

Sample location coordinates	*Bacillus* isolate codes	Total no. distinct colonies selected	No. isolates showing any antagonism	Best isolates with antagonistic potential
Morwatshetlha (W) (25.8842° S, 25.5089° E)	MOREBS1.1,2,3,5, … 20	20	2	*MORWBS1.1
Klipirani (25°52'60" S and 27°25'60" E)	KLIBS2.1,2,3,5,... 20	20	2	*MARBS4.6
Harbeestlaagte (A) (26°11'44.2"S 25°24'25.0"E)	HARABS3.1,2,3,5, … 20	20	2	
Maretsane (26°08'58.7"S 25°25'27.3"E)	MARBS4.1,2,3,5, … 20	20	3	
Vergelee bray (A)(25°46'39.5"S 24°11'26.3"E)	VERABS5.1,2,3,5, … 20	20	1	*VERBS5.5
Morwatshetlha (E) (25.8842° S, 25.5089° E)	MORWBS6.1,2,3,5 ,… 20	20	4	*MOREBS6.3
Vergelee bray (B) ((25°46'32.6"S 24°11'22.5"E))	VERBBS7.1,2,3,5, … 20	20	2	
Molewane (25.8000° S, 25.7333° E)	MOLBS8.1,2,3,5, … 20	20	4	*MOLBS8.5 and *MOLBS8.6

Harbeestlaagte (B) (26°11'44.2"S 25°24'25.0"E)	HARBBS9.1,2,3,5, … 20	20	3	
North West University (25°49'16.8"S 25°36'52.8"E)	NWBS10.1,2,3,5, … 20	20	3	*NWBS10.5

3.4.1 Presumptive selection and identification of bacterial isolates

All the 200 isolates selected from the HiChrome *Bacillus* agar according to manufacturer's identity descriptions (Table 3.1b) were Gram positive, oxidase positive and catalase positive. A large portion of the isolates showed cultural characteristics that resembled strains *Bacillus subtilis* because of their light green to green colonies on the agar. Isolate BS10.5 showed a unique cultural characteristic that was not included in the manufacturer's identity description (Photo 3.1) as such it was not presumptively assigned to a particular specie of *Bacillus*. Isolate BS8.5 and BS8.6 showed characteristics resembling strains of *B. thuringensis*. Isolate BS4.6 and BS4.3 had close resemblance with *B. cereus* while BS6.3 shared close resemblance with *B. pumilis*. Characteristics that resembled *B. coagulans* were seen in isolate BS1.7, BS2.7 and BS3.5.

Table 3.2: Colonial characteristics of rhizobacteria antagonist strains in HiChrome *Bacillus* agar

Organisms (ATCC)	Growth	Color of Colony
B. subtilis (6633)	+/++	light green to green colonies
B. cereus (10876	+++	light blue, large, flat colonies with blue center
B. thuringiensis (10792)	+++	light blue, large, flat colonies with irregular margins
B. megaterium (14581)	+++	yellow, mucoid colonies
B. coagulans (7050)	+++	pink, small, raised colonies

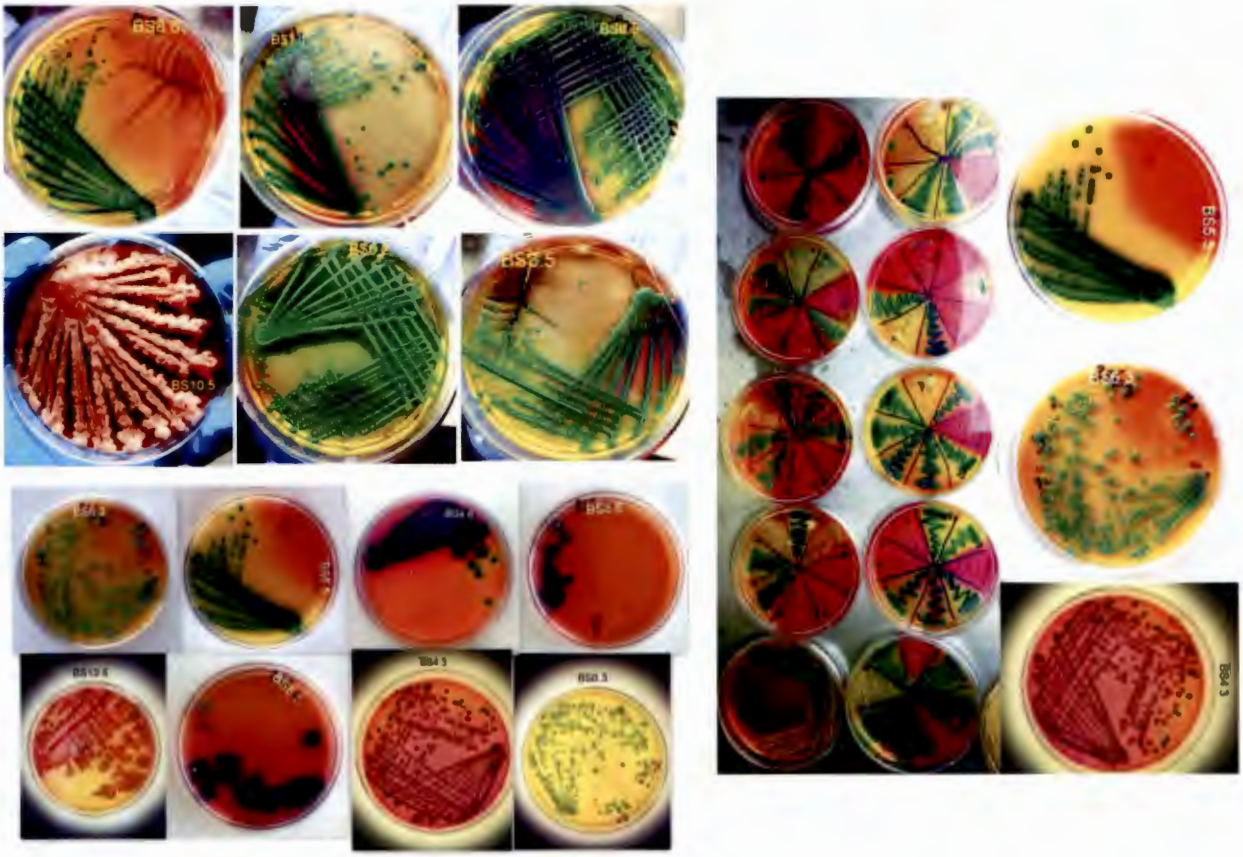


Photo 3.1: Colonial characteristics and the presumptive identification of the isolated *Bacillus* strains on HiChrome *Bacillus* agar.

3.4.2 *Bacillus* inhibition of *F. graminearum* mycelia

Results of the antagonistic activity of the *Bacillus* isolates against the two *Fusarium* pathogens (Table 3.3, 3.4 and Photo 3.2) showed that higher inhibition rates were seen in condition 1 (antagonist inoculated on PDA three days before pathogens) and 2 compared to condition 3 (see 3.2.4.2) were fungal mycelia was inoculated on the PDA plug three days before the antagonists were inoculated.

Table 3.3: Percentage inhibition of *F. graminearum* mycelia by *Bacillus* isolates

	Condition (% inhibition zone)			
Treatment	1	2	3	mean
BS1.1	42.67	46.67	66.67	52.00b
BS10.5	54.67	42.67	77.33	58.22a
BS2.7	42.67	41.33	56.00	46.67cd
BS3.5	45.33	41.33	68.00	51.56b
BS4.3	40.00	38.67	44.00	40.89f
BS4.6	42.67	38.67	44.00	41.78ef
BS5.5	36.00	30.67	58.67	41.78ef
BS6.2	32.00	26.67	69.33	42.67ef
BS6.3	36.00	33.33	65.33	44.89de
BS8.5	45.33	46.67	54.67	48.89bc
BS8.6	40.00	37.33	70.67	49.33bc
mean	41.58b	38.55c	61.33a	
ANOVA				
Treatment (T)	***			
Condition (C)	***			
T x C	***			

Values are means and standard error of four replicates of *in vitro* antagonistic activity of selected *Bacillus* isolates against *F. graminearum*; values having the same letters are not significantly different according to Duncan's least significant difference test at $P \leq 0.05$. 1, 2 and 3 represent conditions of inoculation (**see 3.2.4.2**).

Table 3.4: Percentage inhibition of *F. culmorum* mycelia by *Bacillus* isolates

Treatment	Condition (% inhibition zone)			Mean
	1	2	3	
BS1.1	37.33	34.67	60.00	44.00e
BS10.5	45.33	38.67	70.67	51.56c
BS2.7	44.00	41.33	62.67	49.33d
BS3.5	45.33	46.67	57.33	49.78cd
BS4.3	45.33	50.67	70.67	55.56ab
BS4.6	53.33	45.33	53.33	50.67cd
BS5.5	45.33	44.00	65.33	51.56cd
BS6.2	37.33	36.00	46.67	40.00f
BS6.3	56.00	50.67	69.33	58.67a
BS8.5	52.00	54.67	62.67	56.44a
BS8.6	50.67	57.33	50.67	52.89bc
Mean	46.55b	45.45b	60.85a	
ANOVA				
Treatment (T)	***			
Condition (C)	***			
T x C	***			

Values are means and standard error of four replicates of *in vitro* antagonistic activity of selected *Bacillus* isolates against *F. culmorum*; values same having the same letters are not significantly different according to Duncan's least significant difference test at $P \leq 0.05$. 1, 2 and 3 represent conditions of inoculation (**see 3.2.4.2**).

Overall, Fcul was more resistant to the antagonist during condition 1 and 2 when compared with susceptibility of Fg during those conditions of treatment. However, the inhibition of Fg was less during condition 3. Applying biocontrol agents as bio-protective coating prior to planting of crop seedlings has been demonstrated in previous reports (Yang et al., 2015). Our result

demonstrates that applying seed previously coated with BCAs during planting could protect plants against onset of fusariosis. Out of the 200 initially screened for antagonism against the fusarium pathogens, 11 isolates showed consistent antagonism and from the 11 isolates isolate BS10.5 exhibited the most consistent and strongest antagonistic activity overall considering the conditions. This significant activity of BS10.5 is especially seen against Fg.

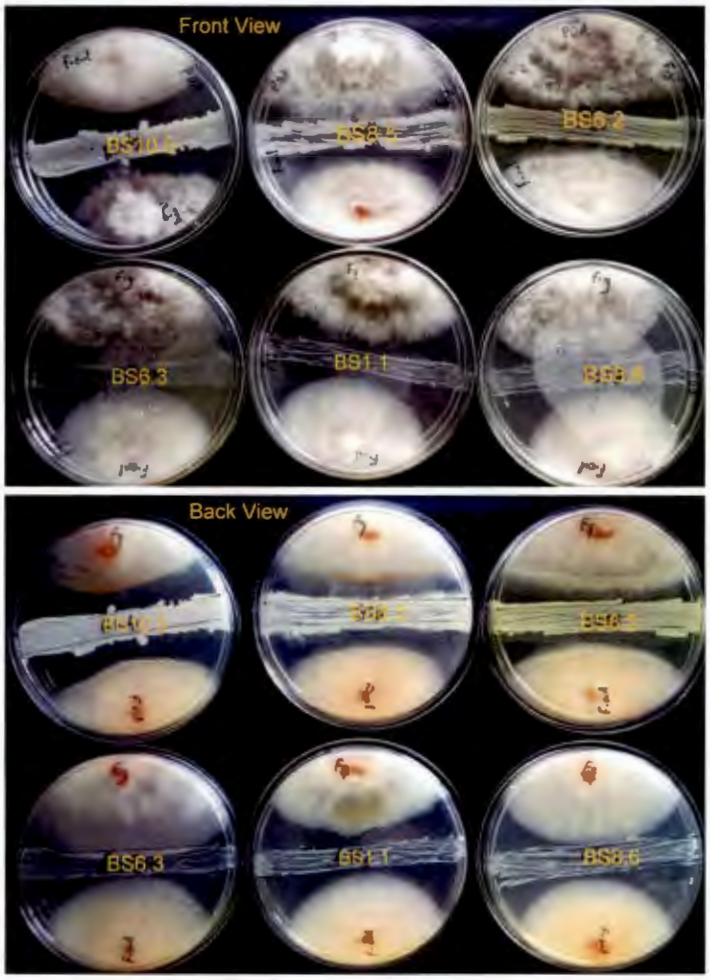


Photo 3.2. Inhibition zones of *F. graminearum* and *F. culmorum* by *Bacillus* isolates in co-culture *in vitro* test.

3.4.3 Sensitivity of *Bacillus* strains to antibiotics concentrations

Multiple antimicrobial resistance by environmental isolates has been reported over years and it has been attributed to increased use of antibiotics during various agricultural practices (Song et al., 2013; Wright et al., 2010; Graham et al., 2016). Although the ability of organisms to resist antimicrobial agents or harbor resistance genes has been argued to confer survival advantage on such organisms (Nwosu et al., 2001; Yilmaz et al., 2003; Wani et al., 2009; Xie et al., 2016), these attributes might be disadvantageous in candidate BCA intended for use in plant growth promotion or crop bio-protection. All the selected *Bacillus* antagonists were sensitive to Rp-Rifampicin; K-Kanamycin; Gm-Gentamycin and C-Chloramphenicol and Nor-Norfloxacin. Isolate BS10.5, BS6.3, BS8.5 and BS5.5 were sensitive to all the antibiotics. However, isolates BS8.6, BS1.1, BS3.5, BS2.7 and BS6.2 all resisted Pg-Penicillin, as no zone of inhibition was observed. Only isolates BS1.1 and BS6.2 resisted Cip-Ciprofloxacin and E-Erythromycin respectively, while BS4.6 and BS4.3 both resisted S-Streptomycin (supplementary figure 3.1/appendix 15). The presence and persistence of drug resistant organisms in the environment still remains a public health threat and the ecological impact of introducing a BCA that harbour drug resistant traits could cause an imbalance in the ecosystem (Etcheverry et al., 2009; Pereira et al. 2009; Streeter et al., 2016). We therefore felt confident to select only *Bacillus* antagonists susceptible to all the antibiotics tested due to the inherent spore forming ability of *Bacillus* strains that enable them to persist in the environment.

3.4.4 Detection of Biosurfactant production

Biosurfactants are biologically surface-active agents with both lipophilic and hydrophilic moieties (Kosaric et al., 1987) that have numerous applications agriculturally, industrially, biologically and medically (Pacwa-Plociniczak et al., 2011). They are produced as virulence

metabolites by the producing organism for competitive advantage and inhibition of biofilm adhesion of pathogenic bacteria (Rivardo et al., 2009). They have also been used commercially to bioremediate contaminated sites and recycle cooking oil wastes because of their low toxicity, high biodegradability and their environmental friendliness compared to chemical surfactants (Li et al., 2016). They act by decreasing the surface and interfacial tension between individual molecules in contact, forming selective ionic pores in membrane bilayers, and they are endowed with diverse biological activities (antiviral, antibacterial, hemolytic, antifungal and anti-insecticidal properties (Mnif et al., 2015). Production of biosurfactants have been reported for many *Bacillus* strains (Kim et al., 2010; Baindara et al., 2013; Plaza et al., 2015; Chen et al., 2016). The multiple test on the seven isolates for detecting biosurfactant production revealed that 4 of the isolates (BS1.1, BS3.5, BS10.5 and BS8.6) are biosurfactant producers (Table 3.5). Although all the isolates showed partial hemolysis on blood agar, isolate BS5.5, BS8.5 and BS6.3 however did not show positive results to the other test carried out (Table 3.5).

Table 3.5: Test for biosurfactant properties of potential isolates

	Test Substance						
	Drop Collapse						
Isolates	Blood Agar[2]	Microplate assay	Vegetable Oil[1]	Motor Engine Oil[1]	Kerosene[1]	Hexadecane[1]	Parafilm[1]
BS1.1	●	●●	●	●	●	●	●
BS3.5	●	○	●	●	●	○	●
BS5.5	●	○	○	○	●	○	○
BS8.5	●	○	○	○	○	○	○
BS10.5	●	●●	●	●	●	●	●
BS8.6	●	●●	○	●	●	●	●
BS6.3	●	○	○	○	○	○	○
Water	○	○	○	○	○	○	○
SDS	●	●●	●	●	●	●	●

1. Drop collapse assay ● = collapse, ○ = no collapse

2. Hemolysis test ● = positive, ○ = negative

3. Drop collapse test: negative (○), spreading (●), comparable with SDS (●); Microplate assay: optical distortion comparable with water (○), optical distortion comparable with SDS (●●); Haemolysis test: b-haemolysis (●), no haemolysis (○).

The positive detection of biosurfactant production in BS1.1, BS3.5, BS10.5 and BS8.6, further correlates the PCR detection of surfactin genes in these isolates, which is not the case with isolates BS5.5, BS8.5 and BS6.3. The stability of the biosurfactants produced under different growth conditions however might need to be determined because production of biosurfactants is dependent on conducive pH, temperature and salinity concentration (Yakimov et al., 1995; Rivardo et al., 2009).

3.4.5 Molecular characterization of *Bacillus* isolates

The PCR analysis carried out against the 7 isolates revealed that each isolate harbored at least a lipopeptide gene. Multiple lipopeptide antibiotic genes were detected in BS10.5 amplicon (Table 3.6) and all the isolates showed a positive result for the Fengycin (Af2-F) primer. Clusters for surfactin genes were detected in all the isolates using the *SrfC* primer. However, only 3 isolates harbored iturin genes and no amplification was detected using the Fengycin (*FenD*), *Ipdc* (Indole Pyruvate decarboxylase) and *Acc* deaminase primers. Single predominant amplicon bands were produced from the PCR products from the gel electrophoresis (Photo 3.3a). Amplification of antibiotic resistant genes for four antibiotics showed that all of the isolates resisted Streptomycin, Tetracyclines (*TetB* and *TetA*) and Sulphonamides. While BS10.5 harbored no resistance genes, all the other isolates harbored ampicillin resistant genes (Photo 3.3b). PGPR susceptible to multiple antibiotics have been reported (Wani et al., 2009). The presence of the lipopeptide antibiotics could be responsible for the antagonistic activities of the *Bacillus* isolates against fungal pathogens and this has been shown in previous reports (Zeriouh et al. 2011; Chen et al., 2016). Further evidence might be needed to fully ascertain this finding. The lipopeptides surfactin, fengycin, bacillomyin and iturins from *Bacillus* spp. have been reported to show broad antimicrobial potentials (Xu et al., 2013; Luo et al., 2011; Jasim et al., 2016), the detection of their genes in the *Bacillus* BS10.5 strain makes it a promising biosuppressor of *F. graminearum* based on the results seen. Numerous members of the *Bacillus* spp., have also been reported to exhibit high biocontrol activities against phytopathogens due to production of compounds belonging to the family of the lipopeptide antibiotics (Vitullo et al., 2012; Cao et al., 2012; Guo et al., 2014).

Table 3.6: Genes detected in the antagonistic *Bacillus* isolates using specific primers sets

Primer set	Isolates						
	BS10.5	BS6.3	BS5.5	BS4.6	BS8.5	BS8.6	BS1.1
Iturin A (*ItuD*)	●	●	○	●	○	○	○
16S (*BacF*/R1378)	●	●	●	●	●	●	●
Surfactins (As1-F)	●	○	●	○	●	●	●
Fengycins (Af2-F)	●	●	●	●	●	●	●
Surfactin (*SrfC*)	●	●	●	●	●	●	●
Surfactin (*sfp*)	●	○	○	○	○	○	○
Fengycin (*FenD*)	○	○	○	○	○	○	○
Bacillomycin D (*BamC*)	●	○	○	○	○	○	○
Ipdc (Indole Pyruvate decarboxylase)	○	○	○	○	○	○	○
Acc Deaminase	○	○	○	○	○	○	○
Strep	○	○	○	○	○	○	○
Amp	○	●	●	●	●	●	●
TetB and *TetA*	○	○	○	○	○	○	○
Sulphonamides	○	○	○	○	○	○	○

● = (Positive) a PCR amplicon of expected size was seen, ○ = Negative

Table 3.7: PCR amplification and target genes

Primer Sequence (5'-3')	Gene Name	Biosynthetic substance	Annealing Conditions	Expected Amplicon size (bp)	Reference
ItuD1f ATGAAGATTTACGGAATTTA *ItuD1r* TTATAAAAGCTCTTCGTACG	*ItuD*	Iturin A	1 min for 55◦C	647	Gond et al., 2015
BacF GGGAAACCGGGGCTAATACCG GAT R1378 CGGTGTGTACAAGGCCCGGGAA CG	16s rRNA	-	90s for 65◦C	1300	Garbeva et al., 2003
Af2F GAATAYMTCGGMCGTMTKGA *Tf1R* GCTTTWADKGAATSBCCGCC	NRPS	Fengycins	30s for 45◦C	443, 452,455	Tapi et al., 2010; Ayed et al., 2014
As1F CGCGGMTACCGVATYGAGC	NRSP	Surfactin	30s for 43◦C	419, 422, 425, 431	Tapi et al., 2010; Ayed et al., 2014

Ts2R ATBCCTTTBTWDGAATGTCCGC C					
FenD1f TTTGGCAGCAGGAGAAGTTT *FenD1r* GCTGTCCGTTCTGCTTTTTC	*FenD*	Fengycin	1 min for 55◦C	964	Gond et al., 2015
sfp-f ATGAAGATTTACGGAATTTA *sfp-r* TTATAAAAGCTCTTCGTACG	*sfp*	Surfactin	1 min for 55◦C	675	Gond et al., 2015
Sur3f ACAGTATGGAGGCATGGTC *Sur3r* TTCCGCCACTTTTTCAGTTT	*SrfC*	Surfactin	1 min for 55◦C	441	Gond et al., 2015
Bacc1f GAAGGACACGGAGAGAGTC *Bacc1r* CGCTGATGACTGTTCATGCT	*Bam C*	Bacillomycin D	1 min for 55◦C	875	Gond et al., 2015
	Strep				
	Amp				
	TetB and *TetA*				

	Sulphonamide			
ipdcF GAAGGATCCCTGTTATGCGAAC C ipdcR CTGGGGATCCGACAAGTAATCA GGC	*IAA*	*Indole pyruvate decarboxylas e*	1700	Kim et al., 2013
ACC4a CAGCAGGAAAAGGATTTGGG ACC4b ACTCCACTGAATTGAACCCG	*acc*	*ACC3 deaminase*	850	Kim et al., 2013

3.4.5.1 PCR amplification and target genes

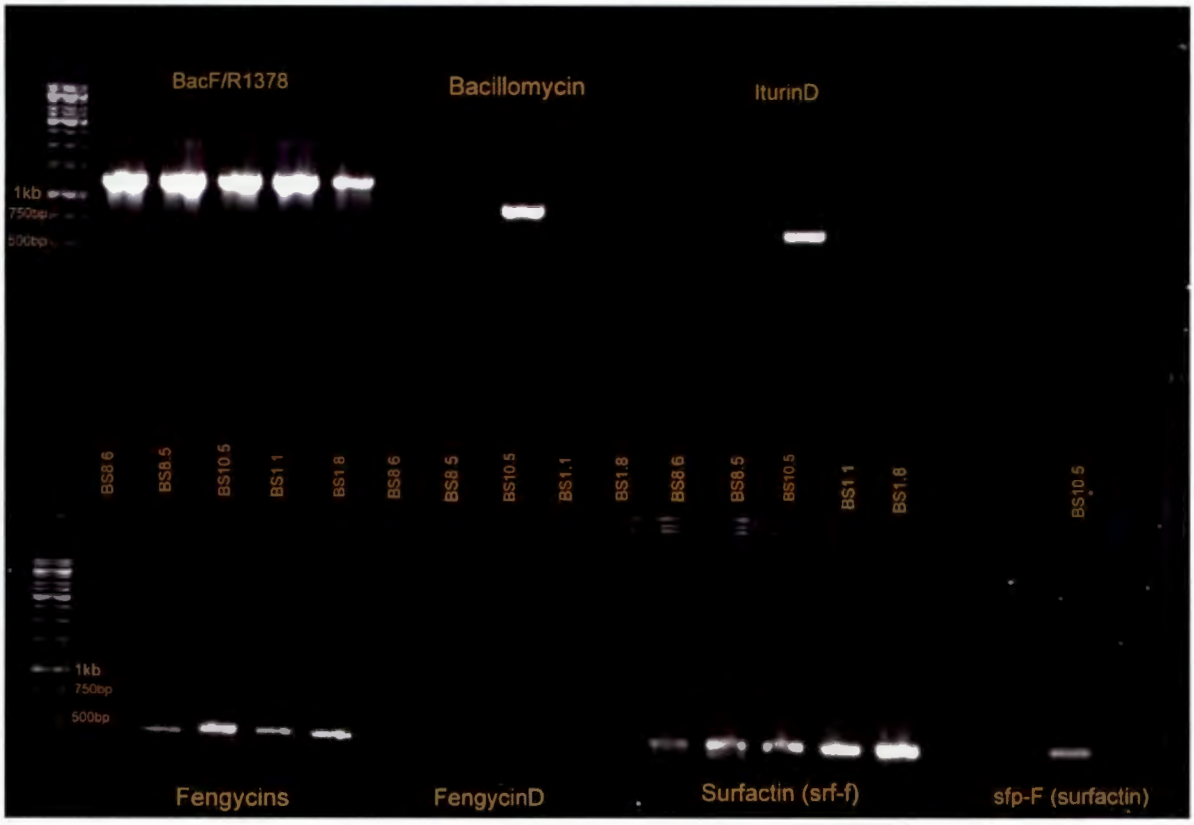


Photo 3.3a: Agarose gel photograph showing amplicons of functional genes in selected *Bacillus* strains with consistent antifungal activity. PCR amplification by degenerate primers: Upper Lane:- *BacF* lane 2-6, *Bacc* lane 7-11, *ItuD* lane 12-16, *sfpF* lane 17-18. Lower lane B:- Fengycins lane 2-6, *FenD* lane 7-11, As1-F lane 12-16, *sfp* F lane 17-19.

Photo 3.3b: PCR amplification of antibiotic resistant genes in PCR product of *Bacillus* strains.

3.4.6 Phylogenetic analysis

Based on the blast search of the partial 16S rRNA gene sequences and submission to NCBI, GenBank accession numbers were assigned to the 7 isolates (Table 3.4). The construction of a phylogenetic tree by MEGA 7.0 software was further used to investigate the phylogenetic positions of the seven isolates using the aligned 16S rRNA MAFFT representative sequences. The positioning were based on evolutionary distances using Neighbour-Joining method (Tamura et al., 2004) and the distance based method inferred the evolutionary relationship using NJ (Neigbour Joining) clustered-based algorithm. In addition, the bootstrap support of the genetic relatedness between 6 of the isolates (BS10.5, BS6.3, BS5.5, BS4.6, BS8.5, BS8.6, BS1.1, BS6.3 and BS1.1) and their nearest neighbors was 100% except isolate BS5.5 that showed 71% close match. The significance of the branches was indicated by a bootstrap for 1000 subsets. Also, isolates BS1.1, BS5.5 and BS6.3 had no direct clustering with any strain on the tree (Figure 3.2). The blast search also correlated with the presumptive identification from the chromogenic agar.

Table 3.8: Blast results of the *Bacillus* isolates partial 16S rRNA gene sequence alignment and identity search on the NCBI webpage

Isolate	Accession no	Blast ID (closest cultured similarity match)	Accession no	Similarity	E- value
KLIBS2.7	MF098606	*Bacillus wiedmannii*	KU198626.1	100	0.0
VERABS5.5	MF098608	*Bacillus cereus*	AE016877.1	100	0.0
MORWBS6.3	MF098609	*Bacillus thuringiensis*	CP020754.1	100	0.0
MOLBS8.5	MF098610	*Bacillus toyonensis*	KJ812450.1	100	0.0
MOLBS8.6	MF098611	*Bacillus thuringiensis*	CP021061.1	100	0.0
MORWBS1.1	MF098612	*Bacillus cereus*	KJ812418.1	100	0.0
NWUBS10.5	KX353617.1	*Bacillus velezensis*	KY694464.1	100	0.0

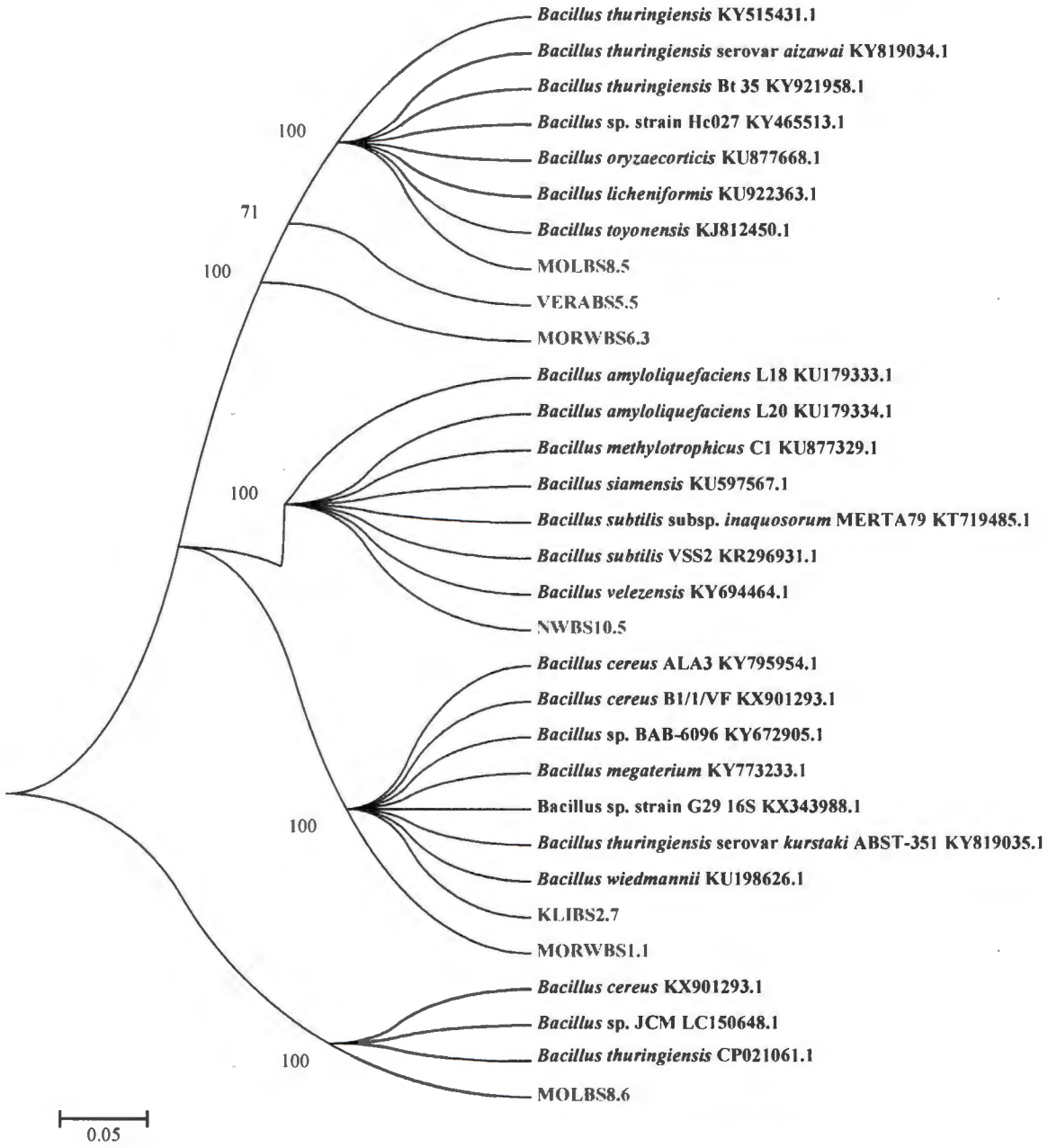


Fig. 3.2: Evolutionary relationships of taxa using Neighbour-Joining method of phylogenetic tree based on partial 16S rRNA gene sequence, showing the phylogenetic relationships between *Fusarium* inhibitors and the most closely related strains from the GenBank. Only values > 50% are shown.

To fully establish the involvement of lipopeptide genes in the biocontrol activity of these *Bacillus* isolates, gene expression studies and mutagenic experiments combined with more robust molecular techniques would need to be done.

3.5 Conclusion

In this study, a total of 200 *Bacillus* isolates from maize rhizosphere were screened, however the seven isolates with strong and consistent antagonistic activity against the *F. graminearum* and *F. culmorum* during *in vitro* dual culture test were selected for further analysis. The antimicrobial secondary metabolites produced by *Bacillus* spp. iturin, fengycin, bacillomycin and surfactin were deduced to be responsible for the antifungal properties exhibited by the isolates. Our goal in this study was to provide a bioprotective *Bacillus* strain for maize against the onset fusariosis. Isolate BS10.5 alone secreted four lipopeptides as seen in the PCR results, had the ability to secrete surfactin as confirmed in the biosurfactant test, and was susceptible to all antibiotics tested. This suggests that the isolate BS10.5 may have a wider application outside our initial aims of using it as a *Fusarium* biosuppressor.

CHAPTER FOUR

SCREENING OF INDIGENOUS MAIZE RHIZOSPHERIC *PSEUDOMONAS* SPP. SUPPRESSING *FUSARIUM GRAMINEARUM* FOR FUNCTIONAL GENES

Abstract

Phytopathogens causing fusariosis or mycotoxicoses in maize are a potential threat to grain quality and availability in affected regions of the world. Native biological agents are becoming ideal options over chemical agents for plant disease management of such in South Africa. Out of 200 native *Pseudomonas* isolates identified from the maize rhizosphere of ten farms in the North West Province of South Africa, 20 were further evaluated for their antifungal activity. Seven isolates (PS2.2, PS9.1, PS1.22, PS6.4, PS1.1, PS7.2, and PS6.8) showed consistent *in vitro* biosuppressive effects against *Fusarium graminearum* and *Fusarium culmorum* with sensitivity to seven antibiotics. Two housekeeping genes *rpoD* and 16S rRNA, were used to obtain partial sequences from the DNA extract of the *Pseudomonas* isolates in order to determine their genetic relatedness. We also used specific primers sets to detect and identify metabolic genes (hydrogen cyanide, phenazine, 2, 4-DAPG, diacetlyphloroglucinol and pyrrolnitrin) responsible for the biosuppressive ability of the isolates. The detection of these nonribosomal peptides synthetases genes in these isolates makes them a probable biofungicide candidate. Antibiotic resistant genes for known antibiotics were not detected in the isolates, this should be an important factor when considering them for field applications. This work emphasizes the significance of the genomic based approach for the identification of biocontrol agents useful for plant disease management.

4.1 Introduction

Interdisciplinary research involving environmental strains of the Gram negative, oxidase positive, catalase negative, aerobic rod shaped, *Pseudomonas* genera have been ongoing by scientist for over 30 decades due to their metabolic diversity and the vast number of organic compounds they are able to utilize. Presently, *Pseudomonas* genus consist of over 150 species, eleven subspecies with newer subspecies being added when novel strains are collected (Bennasar et al., 2010; Arnau et al., 2015; Zhang et al., 2015). The genus has been grouped into nine major categories *P. syringae*, *P. fluorescens*, *P. oleovorans*, *P. lutea*, *P. anguilliseptica*, *P. putida*, *P. straminea*, *P. stutzeri* and *P. aeruginosa* and many of the species within the groups have been of immense benefit to plant growth promotion research, biocontrol studies, and the biofertilizer industry (Raaijmakers et al., 1997; Frapolli et al., 2007; Gomila et al., 2015; Yanes and Bajsa 2016). The Multilocus Sequence Analysis (MLSA) has proved very efficient in the taxonomic division of the genus (Mulet et al., 2012 and Gomila et al., 2015). Fluorescent *Pseudomonas* strains have contributed to the disease suppressiveness of soils, and when used as microbial inoculants have protected plants from infection by soil-borne phytopathogens (Michelsen et al., 2010). They employ protective mechanisms not limited to competition, antibiosis, and induced systemic resistance against plant pathogens by producing diverse antimicrobial secondary metabolites (Raaijmakers et al., 2006; Pliego et al., 2011; Santoyo et al., 2012; Perez-montano 2014). Plant associated *Pseudomonas* strains have been reported to secrete lytic enzymes, antibiotics (phenazines, pyrrolnitrin, 2, 4-diacetylphloroglucino Phl), pyoluteorin (Plt)), siderophores, cyclic lipopeptides (amphisin, viscosinamide, and tensin), volatiles and hydrogen cyanide (HCN) (Raaijmakers et al., 2008; Yanes et al., 2012). The role of these antimicrobial metabolites in the ability of several *Pseudomonas* spp. to protect crops have been well elucidated (Hassan et al.,

2011; Hernandez et al., 2014; Muller et al., 2016). The growing environmental threat due to the use of non-biodegradable chemical fungicides and economic loss generated due to reduced crop yield has pushed scientists to seek for supplementary or alternative means of crop protection. The roles of rhizospheric *Pseudomonas fluorescens* and *P. chlororaphis* strains in alleviating crops diseases caused by fungal pathogens such as *Fusarium verticillioides*, *Gaeumannomyces graminis* var. *tritici*, Colletotrichum acutatum, *Meloidogyne Javanica*, and *Sclerotinia sclerotiorum* have gained considerable attention in the past years (Weller et al., 2007; Hassan et al., 2011; Cordero et al., 2012; Vacheron et al., 2016). Furthermore, application of fluorescent pseudomonads directly to seed or as soil inoculants has been documented to yield excellent biocontrol activities (Lagzian et al., 2013 and Hernandez et al., 2014). In this present work, we identified maize root associated pseudomonads that have the potential of suppressing the growth of the fungal pathogen *Fusarium graminearum in vitro*. Members of the *Fusarium graminearum* species complex (FGSC) have been implicated in fusariosis outbreaks affecting small cereal grains worldwide. In addition, *F. graminearum* affects maize yield in South Africa. We characterized the *F. graminearum* suppressing, native *Pseudomonas* isolates for the production of antimicrobial secondary metabolites and we employed two house-keeping genes to determine their phylogenetic relatedness.

4.2 Materials and Methods

4.2.1 Sampling area

Rhizosphere soil samples were collected randomly in no particular order from ten maize farms at harvest time. Geographic location of the sampling sites covers 28,206 km^2 area.

Temperature ranges between 17°C-31°C during the summer and between 3°C-21°C during the winter with an average rainfall of 360 mm.

4.2.2 Sample collection from rhizosphere

Rhizosphere soil samples were collected from ten different maize farms at harvest time. Depending on the width of the maize plot and in no particular order, 20-30 g rhizospheric soil were sampled randomly from four maize rows 15 m-25 m apart in each plot from the ten maize farms in the North West Province of South Africa. Maize plants were harvested and the roots shaken manually to remove the loosely attached soil. The soil adhering to the roots of the plants was considered as the rhizosphere soil. The soil samples taken in each plot were pooled for each location before analysis so that one soil sample were obtained per plot. Thus, a total of 10 different soil samples were obtained from the different maize plots.

4.2.3.1 Selective and differential isolation of *Pseudomonas* spp. from rhizosphere soil sample

Five grams of each soil sample was inoculated in 45 ml of LB broth and incubated for 16 hr with continuous shaking at 150 rpm in the incubator at 35°C after which a calibrated inoculating loop was used to streak on the surface of 20 *Pseudomonas* agar (P1852 *Pseudomonas* agar (for Fluorescein, Sigma Aldrich) plates with cetrinix supplement (Sigma Aldrich C8721). Ten isolates were randomly selected from each plate based on the distinct characteristics exhibited on the selective-differential agar used for culture. Agar and supplement was prepared according to manufacturer's instructions. Due to the selectivity of the *Pseudomonas* agar, all colonies appearing on this medium were eligible for random picking as *Pseudomonas* spp.

F. graminearum and *F. culmorum* were kindly provided by Dr Claire Prigent Combaret (UMR CNRS 5557) Microbial Ecology of Lyon. University Lyon 1, France and Prof Cristina Cruz

CE3C, Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências da Universidade de Lisboa, Portugal and they were maintained on Potato Dextrose Agar (PDA) plate.

4.2.3.2 Gram staining, oxidase tests and catalase activity

To presumptively ascertain the genera of the selected isolates, Gram staining was performed using a Gram-stain kit (HiMedia). Oxidase activity was determined using 1% (w/v) N, N, N', N'-tetramethyl-1, 4-phenylenediamine dihydrochloride. Catalase activity was confirmed by adding 3% (v/v) hydrogen peroxide solution to colonies grown on LB agar. The isolates were maintained at −80°C in Luria–Bertani (LB) broth with 15% glycerol (v/v) and a 15ml LB broth or agar slant was kept at 4°C as working culture.

4.2.4 Rapid *in vitro* prescreening of large numbers of *Pseudomonas* isolates for antagonistic activity against *F. graminearum.*

4.2.4.1 Preliminary antagonistic activity

Preliminary detection of the antagonistic activities of the 200 *Pseudomonas* isolates against *F. graminearum* was carried out by multiple confrontation dual culture tests. A 5 mm diameter plug from an actively growing (7-day-old) mycelial culture of *F. graminearum* was placed in the center of freshly prepared PDA plates (90 mm). From the 200 *Pseudomonas* isolates initially selected, six fresh colonies from 24 hr LB agar culture were circularly streaked (equidistance 1.5 cm) along each PDA plate at a distance of 1.5 cm from the edge of the plate using a sterile inoculating loop. Control plates consisted of *F. graminearum* placed on PDA alone. The plates were further incubated at 28°C for 7 days. Thereafter, only isolates exhibiting strong inhibition were selected for further antifungal confirmatory tests.

4.2.4.2 Confirmatory *in vitro* antifungal test

Plates were prepared as described above, however antagonism was carried out against two fungal pathogens (*F. graminearum* and *F. culmorum*) in three ways. (a) Single bacterial antagonist pre-inoculated at the center of PDA 3 days before both fungal agar plugs were inoculated on opposite sides, (b). Single bacterial antagonist streaked at center of plate while simultaneously inoculating both fungal agar plugs on opposite sides, (c). Fungal agar plugs pre-inoculated 3 days on opposite sides of PDA plate after which single bacterial antagonist were streaked at the center. The pseudomonads with strong inhibition zones around their streaks against the two pathogens in the screening plates were selected for further characterization. The antagonistic effect was determined by measuring the zones of inhibition (mm). The percentage of growth inhibition was calculated using the formula

$$PGI = [(C1 - C2)/C1] \% 100$$

where PGI is the percentage of growth inhibition, C1 is the control mycelia area of uninhibited fungi, and C2 is the distance of the bacterial colony to the growing edge of the fungal mycelia. Experiments were repeated three times and the values were recorded as the means of three replicates.

4.2.5 Susceptibility of rhizobacterial antagonists to antibiotics

Intrinsic sensitivity of the isolates to seven antibiotics (E-Erythromycin (15µg/disc); TE-Tetracycline (30µg/disc); AMP-Ampicillin (25µg/disc); S-Streptomycin (25µg/disc); AML-Amoxycillin (20µg/disc); CN-Ciprofloxacin (5µg/disc); C-Chloramphenicol (25µg/disc)) at the specified concentrations was done according to previous established protocols (Khalifa et al., 2016). An overnight culture of each isolate was homogeneously spread on the Muller Hinton (MH)

agar plates and antibiotic discs were aseptically placed on the plates. Plates were observed for inhibition zones around the antibiotic discs after 48 h incubation at 30°C. Strains sensitive to the antibiotics tested at concentrations indicated showed a clearing zone. The actual diameter of the zone of inhibition was calculated by subtracting the diameter of the disc from the total diameter (Khalifa et al., 2016) and this was recorded for statistical analysis. The experiment was done in triplicate.

4.2.6 Screening for biosurfactant production

4.2.6.1 Hemolysis test/Blood agar test

Isolates were subjected first to hemolysis test; isolates with biosurfactant producing capability can lyse erythrocytes. A colony loopful from fresh cultures of each isolate or 20 µl of each fresh culture in LB broth was taken and streaked on Blood Agar plates (HiMedia, India). Plates were incubated from 48 to 72 h at 37°C (Chakraborty et al., 2014). The plates were then observed to see the presence of a clear zone around the colonies. However, a clearing zone around the bacterial colony on blood agar is not always confirmatory for biosurfactant production (Youssef et al. 2004; Hazra et al. 2011). We further conducted other confirmatory tests.

4.2.6.2 Drop collapse assay

To determine the production of biosurfactant compounds, a modified ''drop collapse test'' applied according to Yanes et al. (2012) was conducted. Briefly, each well of a 96 well plate lid was coated with 2 µl of test substances consisting of vegetable oil, motor engine oil, kerosene, hexadecane, parafilm and equilibrated for 2 hr. A cell free supernatant from an overnight LB broth culture of each isolate was prepared by filtering through 0.22 µm. A 5 µl drop of the cell-free supernatant of each isolate was then placed in the center of the coated well. The result was

determined visually after 1 min. If the drop remained beaded the result was scored as negative, and if the drop collapsed, the result was scored as positive. Water and LB broth were used as negative controls of the media. Each treatment was repeated three times.

4.2.7 Extraction of genomic DNA

Genomic DNA was extracted from overnight culture of selected isolates using the Zymo Research ZR Soil Microbe DNA Miniprep genomic isolation kit (Epigenetics) following the manufacturer's procedure. DNA quantity and quality was assessed with spectroscopic methods using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA). The presence of DNA in the eluted solution was confirmed on 0.8 % (w/v) agarose gel and was used as the template for polymerase chain reaction (PCR) analysis.

4.2.8 Molecular characterization and biosynthetic gene screening of *Pseudomonas* isolates

Comparative identification of the presumptive *Pseudomonas* isolates was carried out using two house-keeping genes, the 16S rRNA gene and the discriminatory *rpoD* gene (Mulet et al., 2010). The efficacy of specific primer sets in detecting major biosynthetic/functional genes specific to *Pseudomonas* spp. from DNA extracts were determined following protocols previously established by Zhang et al. (2006) and Kim et al. (2013) with a 25µl reaction mixture containing 1.5-2.5 µg of template DNA; 1µl of primer, 12.5µl OneTaq quick load 2X master mix with standard buffer (NewEnglandBiolabs NEB), 9.5-10.5µl nuclease free water in PCR thermocycler. All primers used in PCR amplifications were synthesized by Whitehead Scientific, Integrated DNA Technologies, and genes targeted during the PCR conditions are shown in Table 4.3. The PCR amplicons were analyzed by electrophoresis in 1% (w/v) agarose gel and the sizes of the bands were determined using 1 kb molecular marker. The gels containing ethidium bromide (10 µg/ml)

were visualized and photographed using a gel documentation system (Gel Doc 2000, Bio-Rad) to confirm the expected size of the PCR products.

4.2.9 Sequencing reaction

Both the forward and reverse primers were used in the sequencing of the purified PCR products. The sequencing was done at Inqaba Biotechnical industrial (Pty) Ltd, Pretoria, South Africa with PRISM TM Ready Reaction Dye Terminator Cycle Sequencing Kit using dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Bio systems, USA) by following the manufacturer's instructions. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analyzed using CLC Main Workbench 7.

16S rRNA and *rpoD* sequences were blast searched on the NCBI GenBank and ENA database. The sequence alignment was corrected manually using BioEdit alignment editor (Hall, 1999) and aligned sequences were analyzed using MEGA 7.0 software (Tamura et al., 2011). The phylogenetic trees were constructed based on the 16S rRNA gene and *rpoD* gene sequences separately using the neighbour-joining (Saitou and Nei, 1987) method. Topological robustness was evaluated by bootstrap analysis (Felsenstein , 1985) based on 1000 replicates.

4.3 Statistical analysis

A Multivariate General linear model was used to analyze treatment means, and inhibition rates. Least significant difference test (LSD), Duncan multiple test and Student-Newman-Keuls (SNK) test were used to compare observed means, pathogen-antagonist relationship, treatment effects and effect of conditions of inoculation using SPSS statistical package programme (version 22) at the significance level of 5%.

4.4 Results and Discussion

4.4.1 Characterization and identification of the isolates

Table 4.1: Geographic sites and numbers of *Pseudomonas* isolates selected from samples collected

Sampling site coordinates	*Pseudomonas* isolate codes	Total no. distinct colonies selected	No. isolates showing any antagonism	Best isolates with antagonistic potential
Morwatshetlha (W) (25.8842° S, 25.5089° E)	MOREPS1.1,2 ,3,5,...20	20	2	*MORWPS1.1 and MORWPS1.22
Klipirani (25°52'60" S and 27°25'60" E)	KLIPS2.1,2,3, 5,...20	20	2	*KLIPS2.2
Harbeestlaagte (A) (26°11'44.2"S 25°24'25.0"E)	HARAPS3.1,2 ,3,5,...20	20	2	-
Maretsane (26°08'58.7"S 25°25'27.3"E)	MARPS4.1,2, 3,5,...20	20	3	-
Vergelee bray (A)(25°46'39.5"S 24°11'26.3"E)	VERAPS5.1,2, 3,5,...20	20	1	-
Morwatshetlha (E) (25.8842° S, 25.5089° E)	MORWPS6.1, 2,3,5,...20	20	4	*MOREPS6.4 and MOREPS6.8
Vergelee bray (B)	VERBPS7.1,2, 3,5,...20	20	2	VERBPS7.2

(25°46'32.6"S 24°11'22.5"E)				
Molewane ((25.8000° S, 25.7333° E))	MOLPS8.1,2,3 ,5,...20	20	4	*MOLPS8.3 and *MOLPS8.6
Harbeestlaagte (B) (26°11'44.2"S 25°24'25.0"E)	HARBPS9.1,2 ,3,5,...20	20	3	HARBPS9.1
North West University (25°49'16.8"S 25°36'52.8"E)	NWPS10.1,2,3 ,5,...20	20	3	-

From the 200 maize rhizobacterial isolates selected from the chromogenic agar few showed yellow fluorescent coloration (Photo 4.1). However, none of the isolates showed the cultural characteristics of the reference strain (*Pseudomonas aeruginosa*) indicated for differential identification by the manufacturer. Isolate PS1.1 and PS1.22 were the only mucoid isolates on the *Pseudomonas* agar. All the isolates tested negative to Gram reaction, but positive to oxidase and catalase tests. Because of the selectivity of the agar used for isolation we tentatively placed the isolates in the *Pseudomonas* genera.

4.4.2 Presumptive selection and identification bacterial isolates

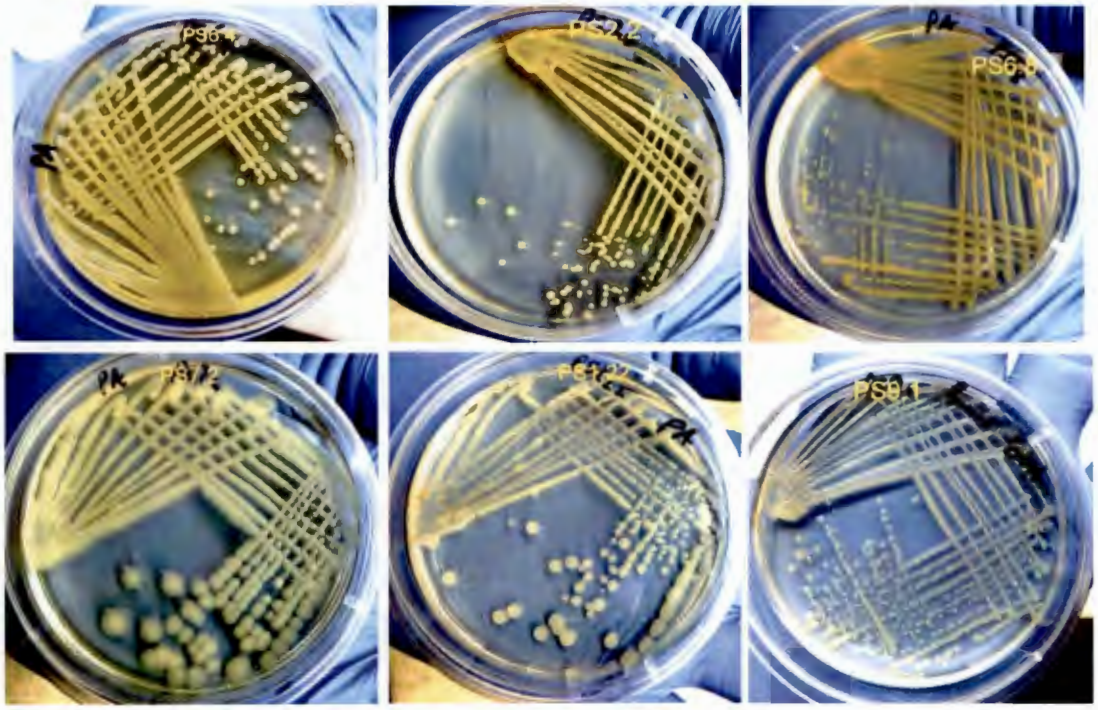


Photo 4.1: Colonial morphology of selected *Pseudomonas* antagonist on *Pseudomonas* agar.

4.4.3.1 Percentage inhibition of fungal mycelia by selected *Pseudomonas* isolates

The results of the antagonistic test against the *Fusarium* phytopathogens revealed that sampling locations 6 and 8 yielded more antagonistic rhizobacterial isolates than other locations sampled. Location 1, 2, 3 and 7 each yielded 2 isolates with strong inhibitory potentials, while sampling locations 4, 9 and 10 yielded 3 antagonists each. Only one isolate was recovered from sampling location 4 (Table 4.1). The most consistent isolates from the preliminary antifungal tests were isolates PS1.1, PS1.22, PS2.2, PS6.4, PS6.8, PS8.3, PS8.6, PS9.1 and PS7.2 (Photo 4.2). From figure 4.1, we see that the antagonists performed better during condition 2 of the inhibition test as the zones of inhibition were relatively high for all the isolates. In condition 1 and 3, the

isolates exhibited varying inhibition rates. Although isolate PS9.4 showed the highest inhibition (<75%) potential against Fg it lacked consistency during the other conditions of antagonism. Isolate PS9.1, however showed consistent inhibition (<65%) against the 2 pathogens during condition 1. Overall, Fcul was more susceptible to the majority of the isolates. Considering that isolate PS9.1 performed better against both *Fusarium* pathogens when pre-inoculated (condition 1), it might be a better bioprotective inoculant for maize against seed borne incidence of *Fusarium*. *In vitro* dual culture prescreening of large numbers of microorganism for antibiosis is easy and inexpensive and it still remains one of the appropriate methods of selecting candidate biocontrol agents from numerous promising strains (Pliego et al., 2011; Shehata et al., 2016).

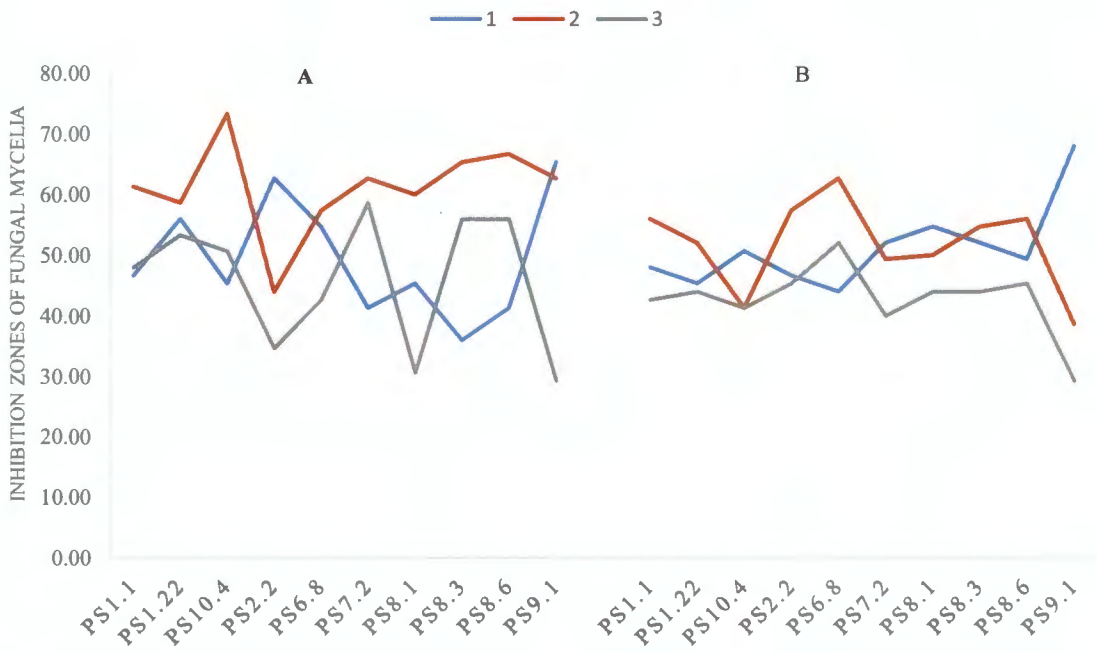


Fig. 4.1: Means of four replications of *in vitro* antagonistic activity of selected *Pseudomonas* isolates against *F. graminearum* (A; standard error = 2.355) and *F. culmorum* (B; standard error = 2.021); Inhibitions are significantly different according to Duncan's least significant difference test at $P \leq 0.05$. 1, 2 and 3 represent conditions of inoculation (**see 4.2.4.2**).

4.4.3.2 Fungal mycelia inhibition by selected *Pseudomonas* strains

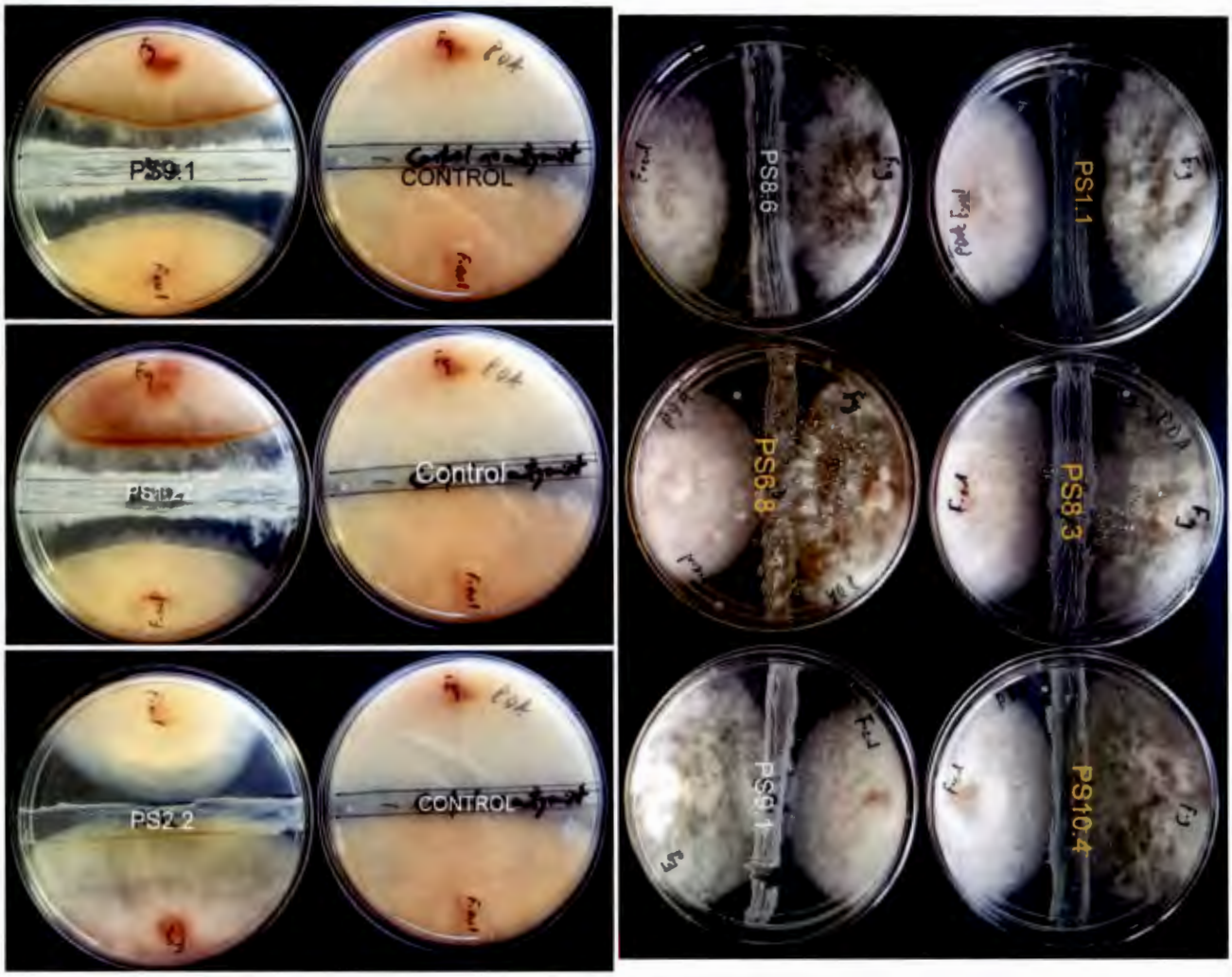


Photo 4.2: Inhibition zones of *F. graminearum* and *F. culmorum* by *Pseudomonas* isolates in co-culture *in vitro* test.

4.4.4 Susceptibility patterns of the *Pseudomonas* to the different concentrations of antibiotics

Antibiotic sensitivity has been used for genotypic characterization over the years (Rashad et al., 2015; Presta et al., 2017). In this study, where zone of clearance by antibiotic is absent, the

microbe is labelled resistant. Here we report the resistance of PS8.4, PS9.1, PS6.8 and PS2.2 to amoxicillin and ampicillin. Both isolates PS8.3 and PS8.1 were sensitive to all the antibiotics, while PS1.1 PS7.2, and PS6.8 alone resisted erythromycin (supplementary figure 4.2/appendix 18-19). Gram negative organisms are naturally resistant to erythromycin (Jensen et al., 2001) therefore the susceptibility to erythromycin by the majority of the selected *Pseudomonas* isolates requires further investigation. Resistance to multiple antibiotics by root zone microbial flora has been reported (Nairn et al., 2002; Yasmin et al., 2009) and this is seen in the antibiotic sensitivity test of our *Pseudomonas* isolates. The survival or competitive advantage antibiotic resistance might confer on candidate plant growth promoting bacteria continues to be topic of debate (Wani et al., 2009; Xie et al., 2016).

In this study 4 (PS7.2, PS1.22, PS8.3 and PS1.1) of our 9 isolates showed biosurfactant properties form the multiple tests carried out (Table 4.2). Soil borne *Pseudomonas* strains exhibiting biosurfactant traits have been reported to antagonize multiple plant pathogens and their capability to utilize diverse substrates for biosurfactant production has been documented (Arutchelvi and Doble 2010; Alsohim et al., 2014; Belgacem et al., 2015; Deepika et al., 2015). Biosurfactants being surface active substances produced by diverse microbes have various applications and their environmentally friendliness, biodegradability, low toxicity, non-hazardousness and inexpensive production cost makes them more attractive than chemically synthesized surfactants (Raaijmakers et al., 2010; Pacwa-Płociniczak et al., 2011; Soberón-Chávez et al., 2011; Rath et al., 2016).

4.4.5 Biosurfactant production screening

Table 4.2: Test for biosurfactant property of potential isolates

Isolates	Test Substances						
			Drop collapse				
	Blood Agar[2]	Microplate assay	Vegetable Oil[1]	Motor Engine Oil[1]	Kerosene[1]	Hexadecane[1]	Parafilm[1]
PS7.2	●	●●	●	●	●	●	●
PS1.22	●	●●	●	●	●	○	●
PS6.4	○	○	○	○	○	○	○
PS6.8	○	○	○	○	○	○	○
PS2.2	○	○	○	○	○	○	○
PS9.1	○	○	○	○	○	○	○
PS1.1	●	●●	●	●	●	●	●
PS8.3	●	●●	●	●	●	○	○
PS8.6	○	○	○	○	○	○	○
Water	○	○	○	○	○	○	○
SDS	●	●●	●	●	●	●	●

1. Drop collapse assay ● = collapse, ○ = no collapse
2. Hemolysis test ● = positive, ○ = negative

Drop collapse test: negative (○), spreading (●), comparable with SDS (●); Microplate assay: optical distortion comparable with water (○), optical distortion comparable with SDS (●●); Haemolysis test: b-haemolysis (●), no haemolysis (○).

4.4.6 Molecular characterization and biosynthetic gene screening of *Pseudomonas* isolates

Pseudomonas spp. harbor several conserved genes responsible for the secretion of secondary metabolites that have beneficial significance to human activities. The conserved genes

amplified during the PCR reaction in the study are described in Table 4.3 and Photo 4.3 (also see appendix). Primers *PsEG*, 70s, 70F, *gryB*, *ITSI* and *Psmn* are all used in combinations or singularly for the Multilocus Sequence Typing (MLST) of *Pseudomonas* spp. for adequate taxonomic or phylogenetic characterization of members within the genus (Frapolli et al., 2007; Mulet et al., 2010; Arnau et al., 2015; Lin et al., 2015; Vacheron et al., 2016). All the isolates showed amplification for the primers *PsEG*, 70F, *gryB*, *ITSI*, *phz*CD, *PCA2* and Psmn. However, only isolate PS6.4 showed amplification for primer 70s. We detected amplification for primers gacA2, *phl*D and BPR3d in isolates PS1.1, PS2.2, PS6.4, PS2.2 and PS6.8 respectivefully. Isolates PS1.1, PS2.2, PS6.4, PS2.2, PS6.8 and PS9.1 produced amplification for the *prn*D primer, while only PS6.4, PS6.8 and PS1.22 produced amplification for hydrogen cyanide primers. Lastly, no amplification was detected for the *acc* (acc3, aminocyclopropane deaminase) and *ipdc* (IAA, indole pyruvate) primers.

This report agrees with the report of Kim et al. (2013). *Pseudomonas* being one of the most studied PGPR (Plant Growth Promoting Rhizobacteria) harbors biosynthetic genes encoding the various antibiotics listed in Table 4.3 (Zhang et al., 2006; Rokni-Zadeh et al., 2011) and many of their rhizobacterial strains from diverse host plants have been reported to exhibit broad spectrum anti-phytopathogenc traits attributed to the presence of these antibiotics (Stockwell and Stack, 2007; Michelsen et al., 2010; Santoyo et al., 2012). The detection of these antibiotics in our *Pseudomonas* isolates makes them valuable strains that should be considered for further biocontrol studies. Cordero et al. (2012) reported the isolation of antibiotic producing *Pseudomonas* strains from maize plants. Our report here remains one of the few documented. Rapidly characterizing

plant associated microbial antibiotic producers will further provide great insight into their roles in enhancing crop health and yields.

Table 4.3: Molecular characterization and genes detected in the antagonistic *Pseudomonas* isolates using specific primers sets

	Isolates								
Primer set	PS1.1	PS1.22	PS2.2	PS6.4	PS6.8	PS8.3	PS8.6	PS9.1	PS7.2
70s	○	○	○	●	○	○	○	○	○
70F	●	●	●	●	●	●	●	●	●
Psmn	●	●	●	●	●	●	●	●	●
ITSI	●	●	●	●	●	●	●	●	●
*gac*A2	●	○	●	●	●	○	○	○	○
PsEG	●	●	●	●	●	●	●	●	●
*phl*D, BPR3d	●	●	●	○, ●	○	○	○	○	○
*phz*CD, PCA2	●	●	●	●, ●	●	●	●	●, ●	●
*prn*D	●	●	●	●	●	○	○	●*	○
*plt*C	○	○	○	○	○	○	○	○	●
*gyr*B	●	●	●	●	●	●	●	●	●
*hcn*BC, FHCN	○	●	○	●, ○	●	○	○	○	○
acc	○	○	○	○	○	○	○	○	○
Ipdc (IAA	○	○	○	○	○	○	○	○	○

phlD = (2,4-DAPG, diacetylphloroglucinol), 70s = (rpoD sigma factor 70), 70 (rpoD sigma factor 70), Psmn (16s DNA), PsEG (rpod sigma factor 70), *phzCD* (phenazine-1-carboxylic acid), prnD (pyrrolnitrin), *gyrB* (DNA gyrase B subunit), *hcnBC* (hydrogen cyanide), *acc* (acc3 deaminase), *ipdc* (IAA, indole *pyruvate* decarboxylase), ● = (Positive) a PCR of expected size was seen, ○ = Negative, ●*= pcr product of unexpected size seen.

4.4.6.1 PCR amplification and target genes

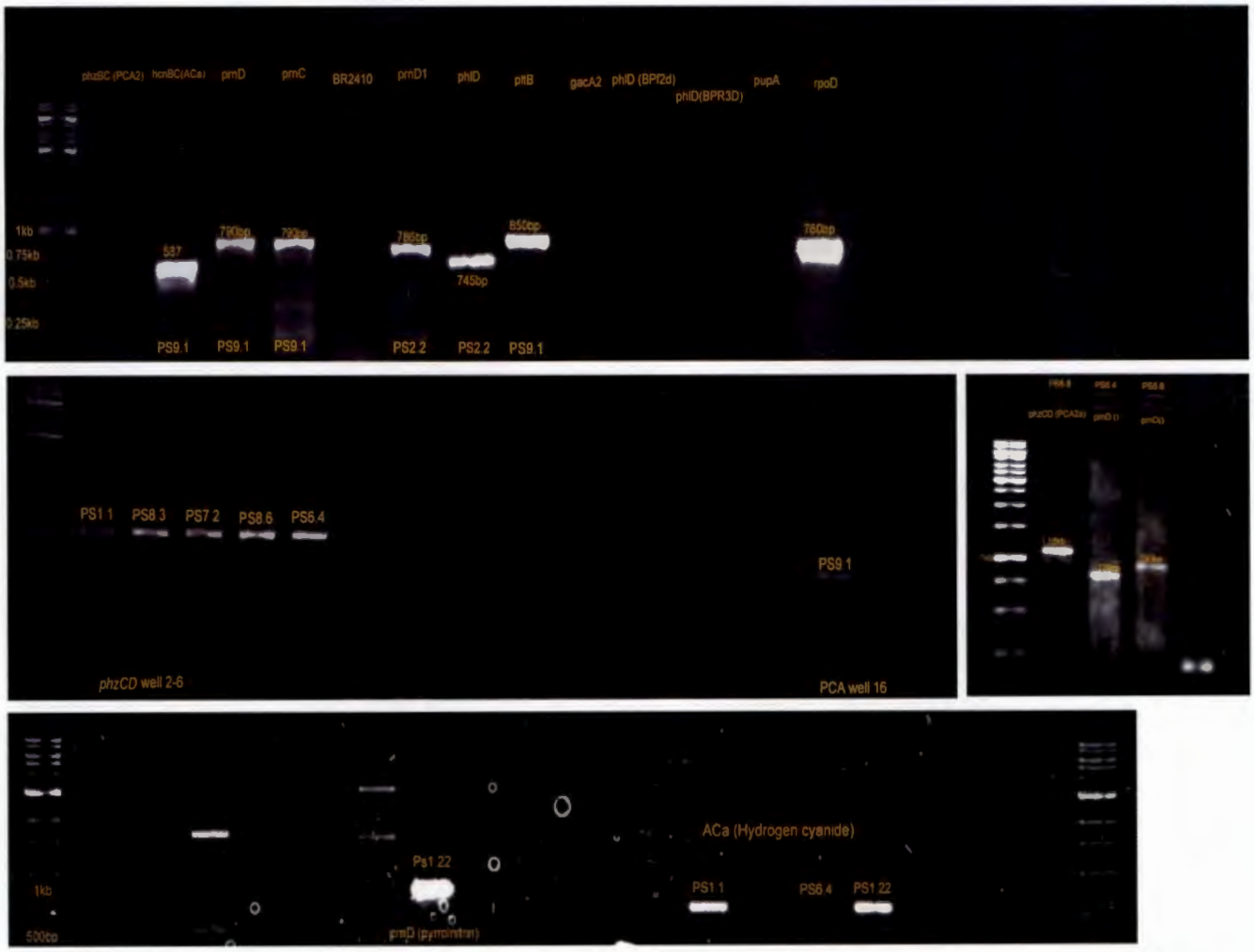


Photo 4.3: Agarose gel photograph showing amplicons of functional genes in selected *Pseudomonas* strains with consistent antifungal activity. PCR amplification by degenerate primers *phlD* = (2, 4-DAPG, diacetylphloroglucinol), 70s = (rpoD sigma factor 70), 70 (rpoD sigma factor 70), Psmn (16s DNA), PsEG (rpod sigma factor 70), *phzCD* (phenazine-1-carboxylic acid), *prnD*

(pyrrolnitrin), *gyrB* (DNA gyrase B subunit), *hcnBC* and *ACa* (hydrogen cyanide), acc (acc3 deaminase), ipdc (IAA, indole pyruvate decarboxylase).

The identification of the *Pseudomonas* isolates was confirmed by computational analysis. BLAST search was conducted in the NCBI database, based on the analysis of partial sequences of the *rpoD* gene and 16S rRNA gene to identify the isolates up to genus and species level (Table 4.3).

Table 4.4: Blast results of the *Pseudomonas* isolates partial *rpoD* gene sequence alignment and identity search on the NCBI webpage

Isolate	Accession no	Blast ID (closest cultured similarity match)	Accession no	Similarity	E-value
VERBPS7.2	MF0985992	*P. putida*	KY938109.1	99	0.0
HARBPS9.1	MF098600	*P. parafulva*	KX756233.1	88	0.0
MOREPS6.8	MF098601	*P. mosselii*	MF627713.1	100	0.0
MOREPS6.4	MF098602	*P. soli*	HF930597.1	99	0.0
KLIPPS2.2	MF098603	*P. putida*	MF427722.1	99	0.0
MORWPS1.22	MF098604	*P. chlororaphis*	KM030051.1	100	0.0
MORWPS1.1	MF098601	*P. soli*	KY351603.1	100	0.0

4.4.7 Phylogenetic analysis

The seven selected *Fusarium* suppressing isolates were subjected to sequencing and phylogenetic analysis. The 16S rRNA and rpoD sequences of the seven isolates were aligned with

multiple reference sequences of the 16S rRNA and rpoD sequences of closely related taxa retrieved from the GenBank data library. The relationships were based on evolutionary distances using the Maximum Composite Likelihood method (Tamura et al., 2004). The distance based method inferred the evolutionary relationship using NJ (Neighbour Joining) clustered-based algorithm. The concatenated NJ revealed the optimal tree of 65.92582460 branch length with 364 positions in the final dataset. Based on the cluster algorithm, NJ tree revealed the percentage of evolutionary relationship within the *Fusarium* suppressing isolates based on the degree of differences among the sequences.

There was no significant difference in the clustering derived from r*poD* and 16S rRNA sequence when analyzed individually. The concatenated NJ revealed that isolates PS6.8, PS1.22 and PS1.1 possessed the highest similarity index of 100% with *P. mosselii*, *P. chlororaphis* and *P. soli* respectively. Equally, PS7.2, PS6.4 and PS2.2 shared very high similarity of 99% with *P. putida* and *P. soli*. PS9.1, however shared 88% similarity with *P. paravulva* as shown in Figure 4.3. The high similarity value expressed by the *Fusarium* suppressing isolates is above 70% borderline of degree of relatedness suggested by Wayne et al. (1987). In addition to this, similarities expressed by these *Fusarium* suppressing isolates with the reference taxa belonging to different species, is as a result of high similarity value exhibited in DNA reassociation values which fall below the 70% threshold values (Stackebrandt et al., 2002). This shows high genetic relatedness that is increasingly reliable because they cannot be wiped out overnight, according to Konstantinidis and Stackebrandt (2013).

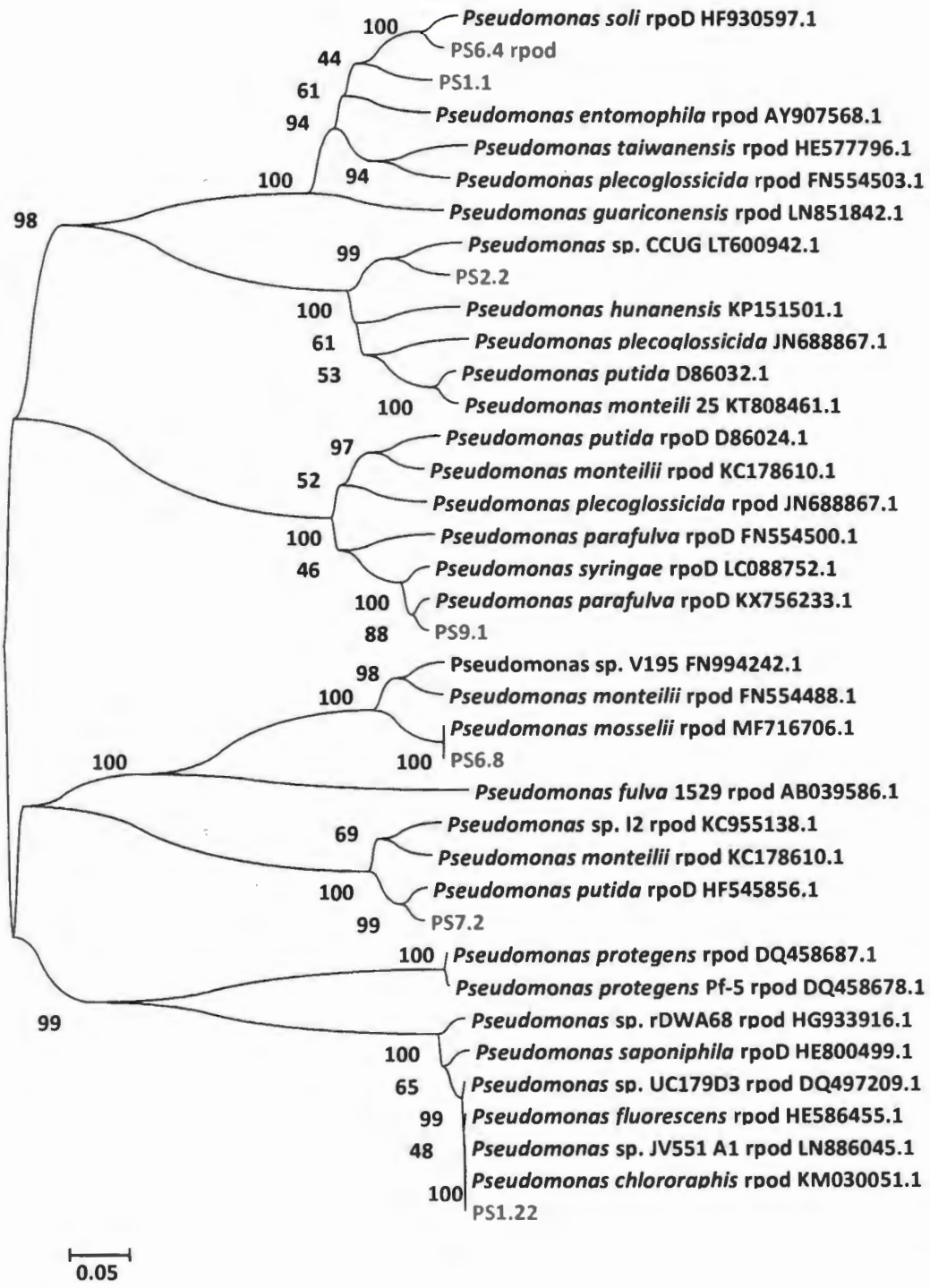

Pseudomonas soli rpoD HF930597.1
PS6.4 rpod
PS1.1
Pseudomonas entomophila rpod AY907568.1
Pseudomonas taiwanensis rpod HE577796.1
Pseudomonas plecoglossicida rpod FN554503.1
Pseudomonas guariconensis rpod LN851842.1
Pseudomonas sp. CCUG LT600942.1
PS2.2
Pseudomonas hunanensis KP151501.1
Pseudomonas plecoglossicida JN688867.1
Pseudomonas putida D86032.1
Pseudomonas monteili 25 KT808461.1
Pseudomonas putida rpoD D86024.1
Pseudomonas monteilii rpod KC178610.1
Pseudomonas plecoglossicida rpod JN688867.1
Pseudomonas parafulva rpoD FN554500.1
Pseudomonas syringae rpoD LC088752.1
Pseudomonas parafulva rpoD KX756233.1
PS9.1
Pseudomonas sp. V195 FN994242.1
Pseudomonas monteilii rpod FN554488.1
Pseudomonas mosselii rpod MF716706.1
PS6.8
Pseudomonas fulva 1529 rpod AB039586.1
Pseudomonas sp. I2 rpod KC955138.1
Pseudomonas monteilii rpod KC178610.1
Pseudomonas putida rpoD HF545856.1
PS7.2
Pseudomonas protegens rpod DQ458687.1
Pseudomonas protegens Pf-5 rpod DQ458678.1
Pseudomonas sp. rDWA68 rpod HG933916.1
Pseudomonas saponiphila rpoD HE800499.1
Pseudomonas sp. UC179D3 rpod DQ497209.1
Pseudomonas fluorescens rpod HE586455.1
Pseudomonas sp. JV551 A1 rpod LN886045.1
Pseudomonas chlororaphis rpod KM030051.1
PS1.22
0.05

Fig. 4.3: Evolutionary relationships of taxa using Neighbour-Joining method of phylogenetic tree based on partial *rpoD* gene sequence, showing the phylogenetic relationships between the *Fusarium* suppressors and the most closely related strains from the GenBank.

Although PCR detection of conserved genes for antibiotic production has been used effectively as an identification tool, it does have limitations. Biocontrol strains have been found not to possess biosynthetic genes for secretion of antibiotics, after carrying our structural and chemical characterization of their cell extracts, even when PCR amplification gave false positive results (Zhang et al., 2006; Muller et al., 2016). However, all the *Pseudomonas* strains with which our selected isolates showed similarities (*P. putida*, *P. parafulva*, *P. mosselii*, *P. soli*, and *P. chlororaphis*), have been shown to be active plant growth promoters or biocontrol strains (Jha et al., 2009; D'Aes et al., 2011; Pascual et al., 2014; Acebo-Guerrero et al., 2015; Liu et al., 2015; Molina- Santiago et al., 2015).

4.5 Conclusion

Our maize rhizobacterial *Pseudomonas* isolates showed biosuppression against the toxigenic fungi *F. graminearum* and *F. culmorum in vitro*. The antibiotic genes identified from their amplicons might be responsible for their antimicrobial properties. A few of the isolates also exhibited ability to produce biosurfactants and other beneficial properties that could make them candidates for biotechnological improvements. Further studies might be necessary to clarify the presence of the detected antibiotics and to determine the *in vivo* antagonistic activity of the pseudomonads.

CHAPTER FIVE

EVALUATION OF THE STABILITY OF MAIZE RHIZOBACTERIA (*PSEUDOMONAS* PS9.1 AND *BACILLUS* BS10.5) STRAINS FOR FIELD APPLICATION

Abstract

The present study evaluated the bio-protective potential of two rhizobacteria (*Pseudomonas* strain PS9.1 and *Bacillus* strain BS10.5) strains on maize against *F. graminearum* proliferation and infection. Reduction of mycelia growth, decrease in fungal spore formation and the ability of the strains to proliferate in the presence of other likely competing microflora were determined *in vitro* and *in vivo*. Results of antibacterial activity against known pathogens showed that the antagonists could have competitive advantage over resident microflora when introduced into new ecosystems or applied as a field biocontrol inoculant. Maize seed assays *in vitro* showed that the antagonists were able to suppress the mycelia and level of spore formation depending on growth medium utilized for the co-inoculation of pathogen and antagonist. The optimal growth conditions for effective cultivation of the antagonist (PS9.1 and BS10.5) was also determined, this information will be valuable when carrying out large scale fermentation of the antagonists. It will also be significant when preparing the mode of delivery of the antagonist for possible field application.

5.1 Introduction

Microflora that inhabit or colonize the rhizosphere can be classified based on the effects they have on plants, and plant roots can serve as portal of entry for both beneficial and pathogenic microorganisms that influence plant growth and development. This zone where root activity influences biological interaction taking place between plant, soil and resident flora significantly is thus referred to as the rhizosphere (Babalola, 2010). The influence of the beneficial microorganisms present in the root zone on plant growth has been studied for years and these beneficial impacts are effected directly and indirectly. The activities of these organisms called rhizobacteria could either result in the stimulation of plant growth or protection of the plant against pathogen attack (Bloemberg and Lugtenberg, 2001). Plant growth promotion involves the direct secretion of plant growth regulators such has auxin, while biocontrol involves the production of metabolites such as siderophores, antibiotics, and hydrogen cyanide, respectively (Bashan and Holguin, 1997; Babalola and Glick 2012).

The production of maize (*Zea mays L.*), one of the most important cereal crops cultivated globally, is adversely affected by members of the *Fusarium graminearum* species complex (FSGC) of which *F. graminearum* Schwabe [teleomorph: *Gibberella zeae* (Schw.) Petch] has been frequently implicated in several worldwide outbreaks (Summerell et al., 2011). Infection of maize by this phytopathogen brings about devastating effects on plant parts, grain quality and yield, resulting in significant economic losses in South Africa where maize is a major staple crop, used by humans for various industrial and agricultural purposes (Lamprecht and Tewoldemedhin 2011; Janse van Rensburg et al., 2015; Mngqawa et al., 2016). The occurrence of *F. graminearum* in maize fields can be detected at both pre-harvest and postharvest, and its infection occurs through

several routes such as systemic infection through the seeds and the movement from the roots to the stalk, sometimes leading to severe rot of the whole plant (Doohan et al., 2003). *F. graminearum* can also infect maize by entering via the silk-channel or through injuries inflicted on the stassels and kernels by insects or birds (Nelson, 1992; Shephard et al., 1996). Furthermore, the mycotoxins (zearalenone, deoxynivalenol (DON) secreted by *F. graminearum* when present in maize and maize-based products pose a health threat to man and their animals (Wang et al., 2011; Zhang et al., 2012; Mngqawa et al., 2016).

The biological control of this notorious plant pathogen has gained worldwide attention in recent years due to the recent outbreaks of the FGSC in different geographic regions of the world (Mullen et al., 2012 and Varga et al., 2015). Also, the continued global awareness on the dependence on inorganic crops, use of chemical fertilizers and agricultural activities that adversely affect the ecosystem has encouraged the introduction of biological disease management practices (Babalola, 2010). Although reports on effective strategies for biocontrolling *F. graminearum* are available, the majority have not shown the effectiveness of indigenously developed biocontrol candidates in the control of localized maize fusariosis. The negative ecological impact of non-indigenous PGPR that are introduced into new ecosystems have been reported in recent times and these include possible repression of non-target microorganisms and disruption of the local ecosystem (Etcheverry et al., 2009; Pereira et al. 2009).

The effectiveness of BCAs in the during field applications is dependent on several environmental factors. Depending on the inherent conditions during pre-harvest or post-harvest of crops, seedling storage conditions, crop diseases or phytopathogen intended to suppress (e.g. endophytes), bioprotectants can either be applied by drenching and coating seeds or spraying of

plant parts (Fravel 2005; Babalola et al., 2007; Pérez-García et al., 2011). Earlier in our studies, we identified through *in vitro* tests maize rhizobacteria from the genera *Bacillus* (BS10.5) and *Pseudomonas* (PS9.1) with bio-suppressive potential against *F. graminearum*. Here we seek to determine the stability and effectiveness of these potential biocontrollers in reducing mycelia production, sporulation and their ability to suppress the pathogenesis of *F. graminearum* during maize pot experiments. The implications of having present the resident flora and non-competing resident flora will be established. This study will show the likelihood of the application of these antagonists in field studies.

5.2. Materials and Methods

The bacteria antagonists used in this study, PS9.1 and BS10.5, were isolated in our laboratory from maize rhizosphere. They were previously identified based on the 16S rRNA and rpoD gene sequence analysis and were assigned the accession numbers MF098600 and KX353617.1 respectively. The fusarium pathogens were provided as gifts (see 3.2.4.1).

5.2.1 Confrontation against bacterial pathogens

Antibacterial activities of the selected rhizobacteria (PS9.1 and BS10.5) were tested against Gram-positive and Gram-negative bacterial strains (Zeriouh et al., 2011). The bacteria pathogens used include BC= *Bacillus cereus* ATCC 10876, EF= *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 25923, *Moxarella cartarrhalis* ATCC 25240 and *Pseudomonas aeruginosa* ATCC 27853. Briefly, 100 µl of overnight cultures of the pathogens (OD0.5:600 nm) were spread on freshly prepared sterile Muller Hinton Agar (MHA) plates (90 mm). Then 80 µl of overnight LB broth culture (OD 0.5:600 nm) of the each rhizobacterial antagonists (PS9.1 and

BS10.5) were loaded in wells made with bottom portion of a sterile 200 µl pipette tip. Plates were incubated at 25+/-2°C for 1-3 days and recordings were taken if zones of inhibition were spotted from the border of the well to the perimeter of visible bacterial.

5.2.2 Optimal growth conditions for isolate PS9.1 and BS10.5

Effect of pH, temperature and salinity changes on selected isolates/antagonists were determined following slight modification of Zhang et al. (2015).

5.2.2.1 Response of PS9.1 and BS10.5 to pH changes

20 ml flasks of LB broth adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using concentrated HCl or 1 N NaOH were sterilized by autoclaving, followed by inoculation with 20 µl of an overnight culture of the isolates (OD 0.5:600 nm). Flasks were incubated at 37°C, 180 rpm for 48 h and optical density (OD) of the cultures were measured at 600 nm using a UV Spectrophotometer (Thermo Spectronic; Merck).

5.2.2.2 Effect of temperature on growth of isolate PS9.1 and BS10.5

Twenty microliter of an overnight culture of the isolates (OD 0.5:600 nm) was inoculated in 20 ml flask of sterilized LB broth and incubated at the temperature ranges of 25, 28, 31, 34, 37, 40 and 43°C. After 48 hr in the shaker incubator (180 rpm), the OD of the isolate at 600 nm was measured using a UV spectrophotometer (Thermo Spectronic; Merck).

5.2.2.3 Tolerance of isolates PS9.1 and BS10.5 to NaCl

Twenty microliter of an overnight culture of the isolates (OD 0.5:600 nm) was inoculated in 20 ml of LB broth supplemented with varying concentrations of NaCl (0.2%, 0.4%, 0.6%, 0.8%,

and 1.0%) at 37°C. After 48 hr in the shaker incubator (180 rpm), the OD of the isolate at 600 nm was measured using a UV spectrophotometer (Thermo Spectronic; Merck).

5.2.3 Surface sterilization of maize seeds

Maize seeds variety DKC 73-72 (200 g) were washed in sterile distilled water to remove any fungicide, soaked for 5 min in a Sodium hypochlorite solution (0.75%), rinsed five times with sterile distilled water and soaked in the fifth wash. The fifth wash was inoculated on nutrient agar to ascertain the sterilization efficiency and surface disinfection was checked by absence of colony forming unit on the NA agar.

5.2.4 Seed germination test

Disinfected maize seeds were subjected to a germination test according to the paper towel method (ISTA 2003) and the percentage germination was calculated according to Abdul-Baki and Anderson (1973). 100 maize seeds were arranged in three 1L beakers (11.8 cm of side) previously wallpapered with towel paper moistened with 10 mL of sterile distilled water. The seeds were again covered with towel paper moistened with 10 mL of sterile distilled water. The beaker was covered and incubated at room temperature for 4 days. After germination, the number of germinated seeds per beaker was counted in order to determine the germination percentage according to the formula

$$GP\% = NG \times 100/TNS$$

where GP = germination percentage, NG=Number of Germinated seed; TNS=total number of seeds.

5.2.5 Maize seed fungal suppression bioassay

5.2.5.1 Bioprotection of maize by PS9.1 and BS10.5 against seed-borne *F. graminearum* and *F. culmorum* incidence

A modified experimental assay of Abd El Daim et al. (2015) was used to determine the bio-protective ability of the bacteria isolates (PS9.1 and BS10.5) on sterile maize grains against *F. graminearum* and *F. culmorum* infection. A loopful of each bacteria isolate from a 24 hr LB agar fresh culture was inoculated in 100 ml LB broth flask and incubated shaking (180 rpm) at 28°C for 24hrs. After an overnight culture, sterile maize grains were submerged in the bacteria LB broth (diluted to OD 0.5:600 nm), incubated at room temperature for 18 hr. Flasks were drained aseptically and air dried under laminar flow for 1 hr and kept for further analysis. A portion of the air dried seeds was inoculated with 8mm agar plugs from 2 week old cultures of either *F. graminearum* or *F. culmorum* and incubated at room temperature for 21 days. Five milliliters sterile distilled water was sprayed on the maize seeds using sterile syringes. Uninoculated seeds and fungal inoculated seeds served as controls. Fungal mycelia suppression was assessed visually and recorded at the end of incubation. After 15 days of incubation, the seeds in the flask were washed with sterile distilled water, mycelia was carefully dislodged and harvested from the maize and a mycelia-spore suspension was prepared by vortexing for 2 mins. The OD of each suspension or mixture was recorded. The percentage reduction in seed-borne incidence of *F. graminearum* and *F. culmorum* in treated seeds was calculated using the formula,

$$\% \text{ Reduction } [(C-T)/C] \times 100$$

where C and T are seed-borne incidence of Fg and Fcul in control and treatments, respectively (Aiyaz et al., 2015).

5.2.5.2 Agar plate seed-borne *F. graminearum* and *F. culmorum* incidence on maize grains

From the air dried bacteria inoculated maize grains above, five grains were equally aseptically removed and placed in a row at the center of PDA agar plates, and then 5 mm agar plugs of both *F. graminearum* and *F. culmorum* were inoculated at opposite sides of the grains/plate. Plates were incubated at 28°C for 7 days in the dark and suppression of fungal mycelia was accessed visually. The numbers of grains that sprouted (with coleoptile) and covered with mycelia were recorded. The length of the radicle/shoot of the sprouted grains was also recorded. Untreated seeds were used as a control. The experiment was done in four replicates and repeated three times (Rahman et al., 2016).

5.2.6 Mycelial mass reduction and spore suppressing capacity of rhizobacteria isolates

In order to determine the effect of the isolate (PS9.1 and BS10.5) treatments on the mass of *F. graminearum* and *F. culmorum* mycelia, 5 mm agar plug of each fungus from 7day old culture was inoculated in 200 ml freshly prepared Tryptic soy broth (TSB) or LB broth and 100 µl of each antagonist (OD 0.5:600 nm) was added to make broth mixtures of antagonist bacteria-*Fusarium* (Dal Bello et al., 2008; Grosu et al., 2015). Control flasks without bacteria (5 mm fungal mycelia in LB or TSB) were also prepared. To ensure continuous homogenization, inoculated flasks were incubated at 28°C for 21 days on a rotary shaker after which the hyphae/mycelia/spores were harvested on Whatman No.1 filter paper. The rate of fungal antagonism by bacterial isolates was determined by measuring both the wet and dry weight of filter paper containing hyphae and spores (filter papers were dried under sterile laminar air flow for 4 hr). The OD of the spore suspensions

collected was also recorded. Three replications of each test treatment were done. The mycelia weight reduction was evaluated according to the following formula:

$$\text{Efficiency (\%)} = [(A-B)/B \times 100$$

where A stands for weight of the filtrate (filter paper with mycelia minus filter paper) from *Fusarium* broths alone and B is the weight of the filtrate (filter paper with mycelia - filter paper) from the *Fusarium*-bacteria antagonist broth mixtures.

5.2.7 Greenhouse experiment

5.2.7.1 Collection of soil for pot experiments

Planting soil was collected from the North West University Animal Science Department agricultural planting area. Sterilization of a portion of the soil was by dry heat for 1 week at 120°C. Sterilized soil was plated on NA to ascertain the sterilization, and sterilization was continued until no growth was observed on the NA plates. Planting pots with dimensions 13cm (diameter) x 10 cm (depth) were filled with 80 kg of both sterilized soil and unsterilized soil up to water-holding capacity. Both sterile and unsterile soil are utilized to compare the persistence and competitiveness of the rhizobacterial antagonists.

5.2.7.2 Pre-germination of maize grains for pot experiments

Two hundred grams disinfected maize grains presoaked in sterile distilled water were placed in 1L beaker previously wallpapered with sterile paper towel moistened with 10 mL of sterile distilled water. The seeds were again covered with sterile paper towel moistened with 10 mL of sterile distilled water. The flask was covered and incubated at 30°C for 5 days after which only pre-germinated seeds with 2 cm callus were used for seed-root dip pot experiment. In this

inoculative release approach, microbial treatments are expected to manifest effect during plant development as they proliferate on and within plant parts (Cook et al., 1996).

5.2.7.3 Seed treatments preparations

From an overnight LB broth culture of PS9.1 and BS10.5, 20 µl of each isolate was transferred into 100 ml of LB broth in a 250 ml Erlenmeyer flask, and cultured for 3days (28°C) with continuous shaking at 150 rpm. Bacteria cells were recovered by centrifugation at 8,000 g for 20 mins and supernatant was discarded. The pellet of each isolate was re-suspended in 100 ml sterile distilled water and concentration was adjusted to OD 0.5:600 nm in sterile solutions of 100 ml LB broth. For single bacterization, 120 sterile pre-germinated maize seeds were submerged in the 100 ml bacteria inoculum (OD 0.5:600 nm) of each treatment, and the mixture bacterization consisted of 120 pre-germinated seeds submerged in 100 ml of bacteria co-inoculation broth (50:50 v/v) at OD 0.5:600 nm. This was incubated for 24 hr with continuous shaking at 100 rpm for homogenization and adherence of bacteria to seeds. Sixty sterile pre-germinated maize seeds were also inoculated in 10^7spores ml^{-1} of *F. graminearum* and *F. culmorum* respectively. The overnight pre-germinated (120) bacterized seeds above were also air dried and sixty grains were aseptically removed and submerged in the spore suspension of each fungal pathogen (10^7spores ml^{-1}). All the treatments (see Table 5.3) were incubated overnight to allow for adherence of inoculum to the seeds. The germinated seeds were then transferred to both soils, with four seeds in one pot, in triplicate. Plants were watered with sterilized water. Uninoculated plants were used as a control and the experimental approach was randomized complete block design with a minimum of three replications.

Furthermore, treatments consisting of 10 ml bacteria antagonist, 10 ml mixtures of both antagonist, and 10 ml of pathogen spore suspensions (10^7 spores ml^{-1}) were applied to the plants using a sterile syringe after one week of seeding in pots according to the treatments listed above. Planting was conducted in three experimental periods. The first experimental period was for 2 weeks in January 2017 after which plants were harvested and growth parameters/disease incidence were observed and recorded. The second experimental period was for 40 days during February 2017 – March 2017 and the third experimental period was for 3 months July 2017 – September 2017. However, during the third experimental period, treatments consisting of 10 ml bacteria antagonist, 10 ml mixtures of both antagonists, and 10 ml of pathogen spore suspensions (10^5 spores ml^{-1}) were applied (following the experimental design and treatments) after 1 week of seeding, using a sterile syringe. Harvesting was done, plantings were evaluated for growth and survival, growth parameters were recorded and all the data was analyzed statistically.

5.3 Results and Discussion

5.3.1 Confrontation against bacterial pathogens

Rhizobacteria strain PS9.1 suppressed the growth of BC, KP and PA at equal percentage, while EF and MC were less susceptible. KP resisted the antagonistic activity of BS10.5 as no zone of inhibition was observed. However, BC, EF and PA were inhibited. PA showed reduced susceptibility compared to BC and EF (Table 5.1).

Table 5.1: Antagonistic potential against known bacterial pathogens

	Bacterial Pathogens				
Test isolates	BC	EF	KP	MC	PA
PS9.1	+ + +	+ +	+ + +	+ +	+ + +
BS10.5	+ + +	+ + +	-	+ + +	+ +

EF = *Enterococcus faecalis* ATCC 29212, KP = *Klebsiella pneumonia* ATCC 25923, MC = *Moxarella cartarrhalis* ATCC 25240, PA = *Pseudomonas aeruginosa* ATCC 27853, BC = *Bacillus cereus* ATCC 10876. + = zone of inhibition, - = no inhibition zone (+ = weak, ++ = medium, +++ = high, represent relative inhibition rates against growth of each bacteria pathogen on the LB medium to the level of 30–49%, 50–69% and ≥70%, respectively.

Promising biocontrol strains and their secondary metabolites showing antibacterial potentials against both Gram positive and Gram negative pathogens have been documented elsewhere (Aunpad and Bangchang, 2007; Arutchelvi et al., 2010; Ayed et al., 2014; Adegboye and Babalola 2016).

5.3.2 Optimal growth conditions for isolate PS9.1 and BS10.5

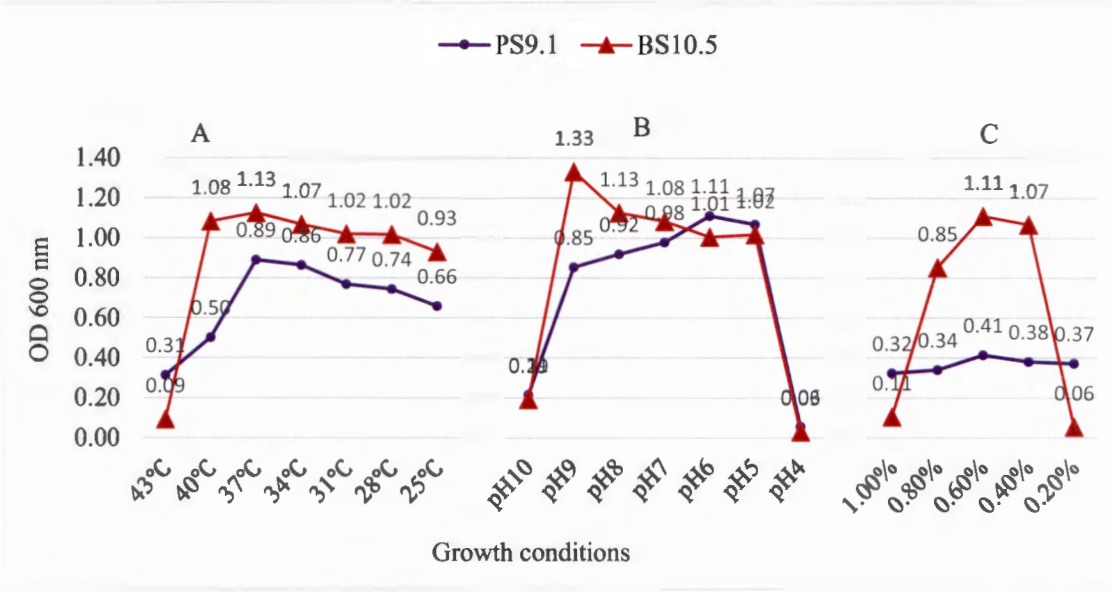


Fig. 5.1: (A) Growth curves of PS9.1 and BS10.5 at different temperatures in LB broth; (B) Growth curves of PS9.1 and BS10.5 at different pHs in LB broth; (C) Effect of NaCl on the growth of PS9.1 and BS10.5 in LB broth.

The results shows that the optimum temperature for maximum growth of the isolate PS9.1 was between 34°C-37°C and BS10.5 grew best between 34°C-40°C. Decline in growth was observed when the temperature was raised above 40°C, which could have been as a result of decreased metabolic activity. Increasing the temperature of growth medium affects the arrangement and stability of bacterial cell wall, ionized chemical moieties and also leads to disruption of the enzymatic activities which consequently slows down the metabolic activities of bacteria (Adamo et al., 2005). Similarly, low temperature also reduced bacterial growth because enzymes are inactivated at this temperature, which decreases the rate of metabolism (Feller, 2013).

Hydrogen ion concentration (pH) is another crucial factor that plays a significant role on the metabolic activities of bacteria. The solubility and ionization state of the functional groups of bacteria cell wall such as carboxylate, phosphate and amino acids are affected by the pH of the bacterial habitat. (Varghese, 2012). The result of the effect of pH on bacterial growth indicated that optimum pH range for the bacteria PS9.1 growth was 6 and for BS10.5, it was 9 while a significant decline in growth was observed when the pH dropped below 5 or increased above 9.

When selecting rhizobacterial candidates intended to be used for biocontrol applications, response to fluctuating abiotic factors such as salt tolerance is another important factor to consider because it affects the rate at which they respire. In the result, isolate BS10.5 showed better salinity tolerance than PS9.1. Although a decrease in the cell density of PS9.1 was observed at the salt concentration of the medium, it grew better at the pH ranges tested with respect to BS10.5, where low salinity resulted in a decrease in cell density, and a relative increase above 0.6% caused a sharp decline in the bacterial growth. Bacteria are able to produce osmolytes like sugar and amino acids to protect themselves against the hypertonic environment created by the salt (Bacilio et al., 2004; Khalid et al., 2017). At a higher concentration of 1.0%, the growth of BS10.5 was greatly reduced as it could not withstand the high osmotic gradient created by the salt.

5.3.3 Seed germination test

$$\% \text{ GP} = \text{NG} \times 100/\text{TNS}$$

$$\% \text{ GP} = 200 \times 100/200$$

During the seed germination test all the seedlings tested sprouted healthily. Two hundred seeds were grown and they all sprouted. Growth parameters were however not recorded for the seed germination test.

Photo 5.1: Germination of maize seedlings prior to *in vitro* and *in vivo* usage

5.3.4.1 Bio-protective capability of isolates on maize grains in reducing mycelia growth and fungal sporulation

There was no significant difference in the bioprotective potential of the antagonists when compared against each other. The reduction of seed-borne incidence of both Fg and Fcul by the isolates was greater than 50% but less that 70%. However, a significant difference was observed between the treatments and control. The treatments related to PS9.1, PS9.1-Fg and Ps9.1-Fcul had higher significant effects on seedling germination compared to the treatments related to BS10.5, BS10.5-Fg, BS10.5-Fcul and fungal treatments. The control seeds with fungal treatments resulted in lowest seedling germination as observed in Table 5.2 and Photo 5.2.

Table 5.2: Efficiency of bioprotective capability of antagonists against fungal seed borne incidence

	Treatments								
Effects	Control	Fg	Fcul	BS10.5	PS9.1	BS10.5-Fg	BS10.5-Fcul	PS9.1-Fg	PS9.1-Fcul
Mean OD nm)	-	3.4	4.0	-	-	1.3	1.9	1.2	1.9
% seedling germination	100	46	48	90	80	60	68	82	96
% reduction of seed borne incidence	-	-	-	-	-	62.7	51.9	62.7	52.6

Photo 5.2: Efficiency of bioprotective capability of antagonists against fungal pathogen seed borne incidence

5.3.4.2 Agar plate bioassay on maize grains

Table 5.3: Agar plate maize seed protection test of selected isolates

Isolates	Grade of inhibition*	No. of seed sprouted/ % germination	No. of seeds covered with mycelium	Average length of radicles (mm)
PS9.1	+++++	2 (40)	0	4.5
BS10.5	+++++	1 (20)	0	3.5
controlA*	-	4 (80)	5	5.0

* Values were graded on a scale from - to +++++ (Grade += 0-5), (- five seeds covered with mycelia, + four seeds covered with mycelia, ++ three seeds covered with mycelia, +++ two seeds covered with mycelia, ++++ one seed covered with mycelia, +++++ no seed covered with mycelia). controlA* seeds not inoculated with bacteria isolates but having fungi inoculated on periphery ends of plates

Maize seeds treated with PS9.1 and BS10.5 suppressed fungal mycelia growth on seed surfaces, while the control seeds was covered with mycelia. The bacteria treated seeds exhibited a healthier vigor compared to the untreated control (Table 5.2 and Photo 5.3). The untreated seeds showed a sign of rot compared to the treated seeds that were covered with mycelia.

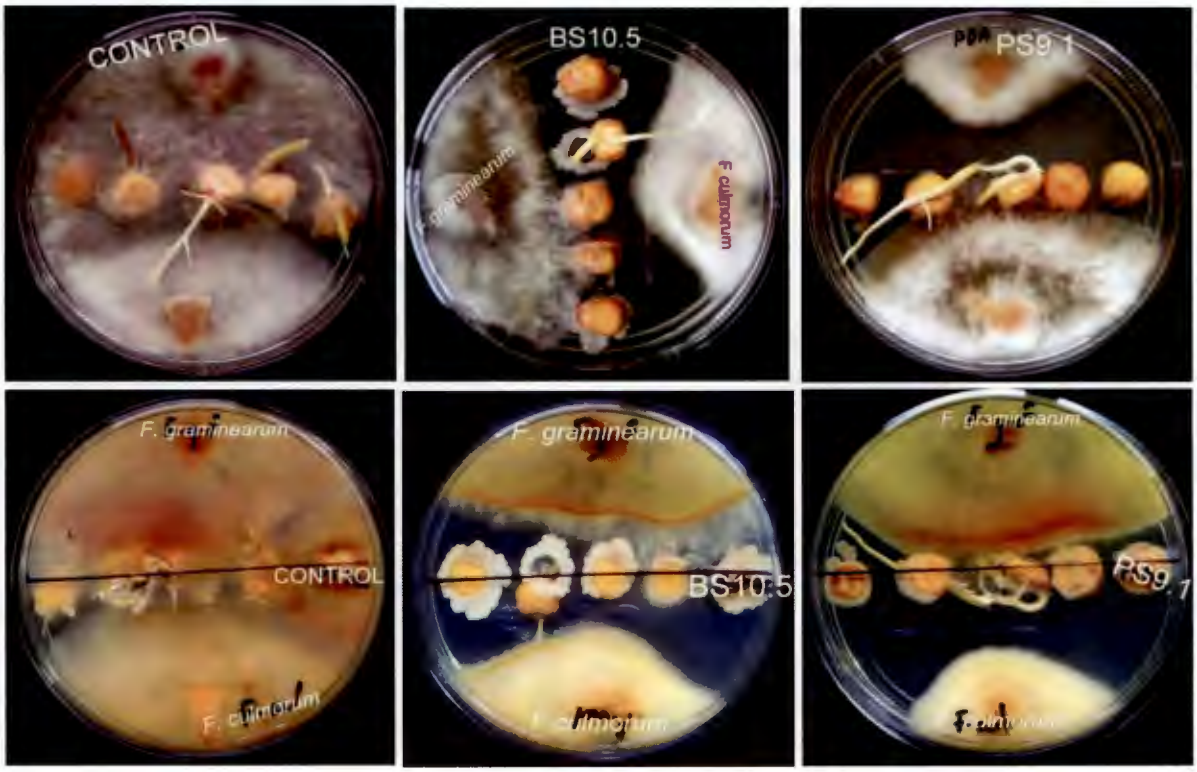


Photo 5.3: Agar plate seed protection test of selected isolates

5.3.5 Mycelial mass reduction and spore suppressing capacity of rhizobacteria isolates in different media

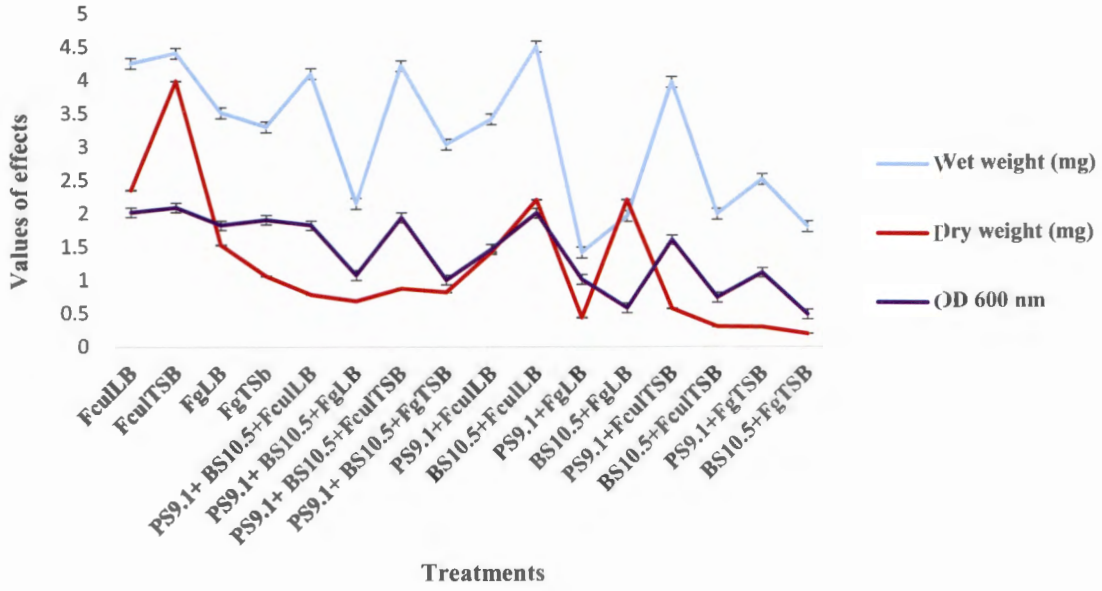


Fig. 5.2: Reduction of fungal mycelia and sporulation after treatments with antagonist in LB broth and TSB. Values represents means of three replicates and effect of treatments are significantly different at P = 0.05 according to Duncan's LSD test.

Co-inoculation of the rhizobacteria strains BS10.5 and PS9.1 with the fusarium pathogens in TSB caused a remarkable reduction in mycelia weight and level of sporulation for both Fusarium pathogens when compared with the control (in LB and TSB) and all other treatments. Co-inoculation of both bacteria with Fg in either LB or TSB however caused decreased mycelia weight and sporulation compared to co-inoculations with Fcul in LB and TSB. Also, Fcul grew better than

Fg in both agar media. Furthermore, the dry mycelia wieght and sporulation of Fcul and Fg was more reduced by PS9.1 in LB than BS10.5 in LB. As reported by Grosu et al., (2015), bacterial mix treatments causes a reduction in fungal inhition rate and this correlates wth our result. Here the treatment combinations having bacteria mix (PS9.1-BS10.5-FculLb, PS9.1-BS10.5-FculTSb, PS9.1-BS10.5-FgLb and PS9.1-BS10.5-FgTSB) showed decreased suppression of fungal sporulation and mycelia weight. This might be due to incompatibility of the two antagonists, which we did not test in vitro in this study. Kolsi et al., (2016), however reported the compatibility of bacteria anatagonist mixes as well as their effectiveness when compared with other treatments employed to bioprotect wheat against *F.graminiearum*.

5.3.6 Greenhouse experiment

5.3.6.1 Pre-germination of maize grains for pot experiments

Photo 5.4: Pre-germinated seeds submerged in the 100ml bacteria inoculum (OD 0.5:600 nm) of each treatment.

Table 5.4: Pot experiment treatment combinations for both soils (sterile and unsterile) used

Soil (Sterile or Unsterile)	A	B	P
Mu/Ms	-	-	-
Mu/Ms	+	-	-
Mu/Ms	-	+	-
Mu/Ms	-	-	+
Mu/Ms	+	+	-
Mu/Ms	+	-	+
Mu/Ms	-	+	+
Mu/Ms	+	+	+

M = Maize; A = PS9.1 inoculant; B = BS1.5 inoculant; P = Pathogen inoculant (*F. graminearum*); Mu = Maize in unsterilized soil; Ms = Maize in sterilized soil

5.3.6.2 Harvest of pot experiments conducted over three experimental periods.

We investigated the bioprotective capability of PS9.1 and BS10.5 during maize germination. The first experiment lasted 2.5 weeks and the plants were harvested at the V4-V5 stage (Photo 5.5). The parameters measured at harvest were wet plant weight, shoot length, root length and dry plant weight. Although plant growth was significantly retarded in the plants with only Fg treatments and roots lacked vigour (M+Ps; M+Pu), no rots or wilting was observed. Also in the non-bacterized plants, the primary and lateral roots were not fibrous despite watering and

the mesocotyl were unhealthy. This could be attributed to Fg systemic infection (Doohan et al., 2003). Although treatments with BS10.5 had better shoot length both in sterile and unsterilized soil, the fresh plant weight of plants without any treatments was significantly higher. In addition, the plants with BS10.5 had longer roots than all the other treatments, from visual observation plants treated with PS9.1 had more fibrous roots and root hairs.

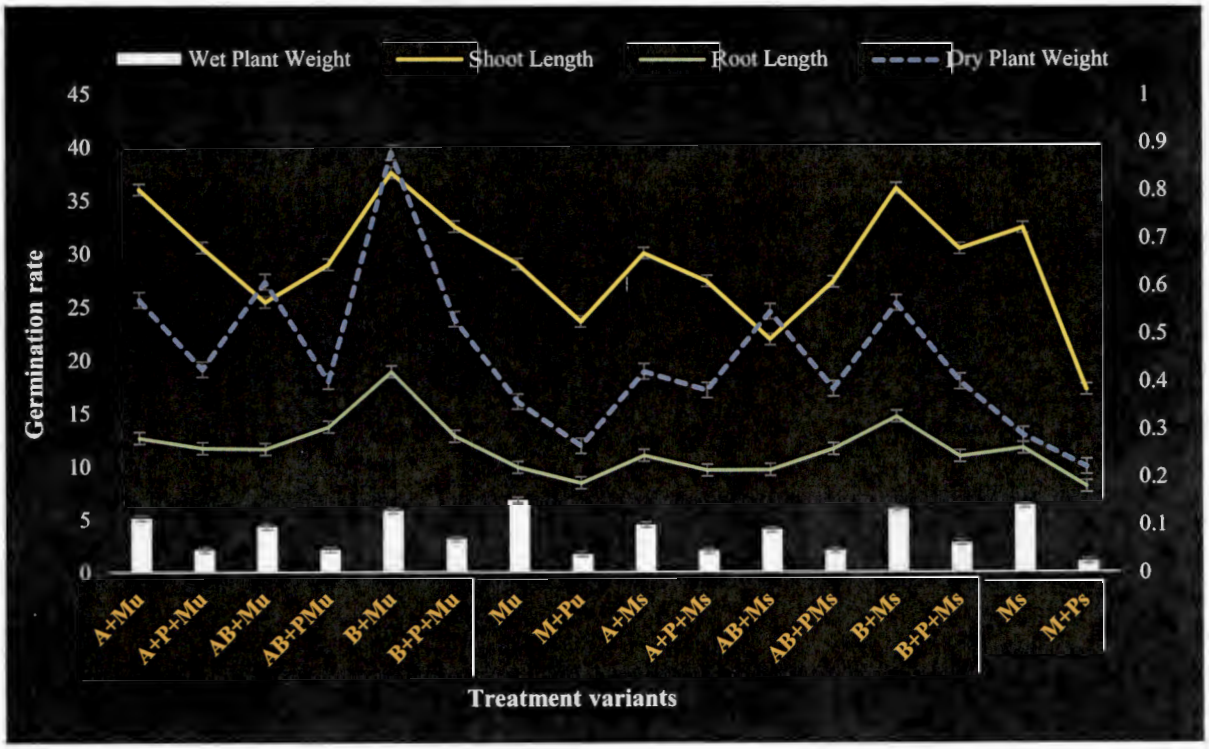


Fig. 5.3: Harvest of first experimental period. All values are the means of three replicated pots from two repeats. Effects of treatments are significantly different at P = 0.05 according to Duncan's LSD test. Wet plant weight, shoot length and root length are on primary horizontal axis, while dry plant weight is on the secondary horizontal axis.

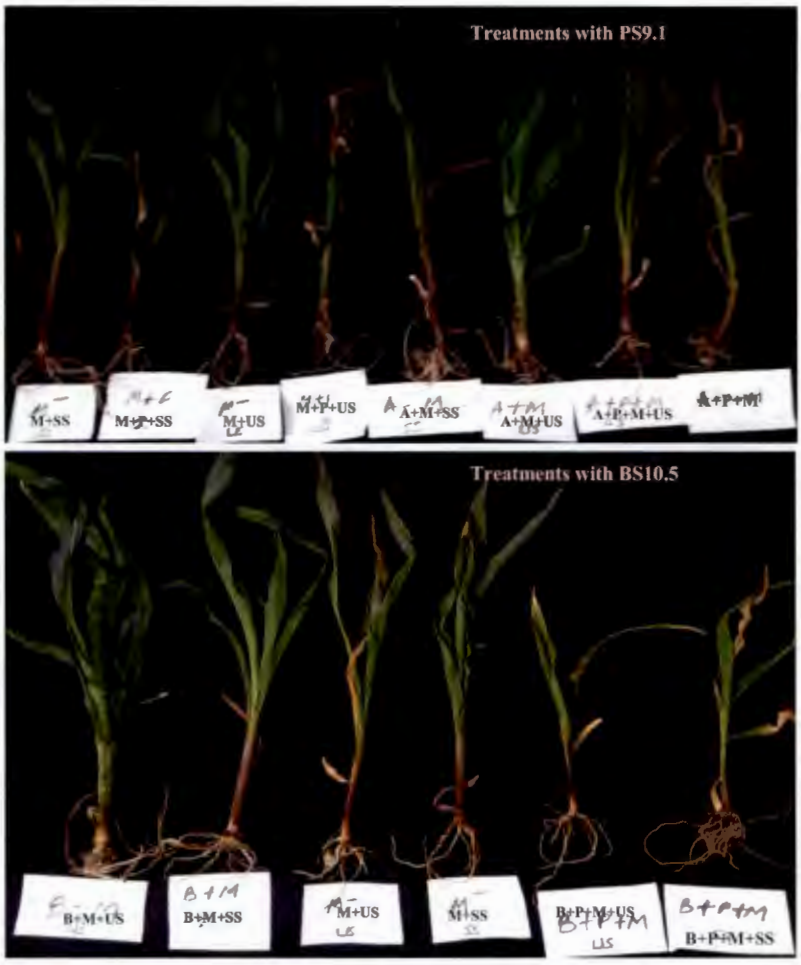


Photo 5.5: Harvest of plantings at V4-V5 stage (2.5weeks) after seeding.

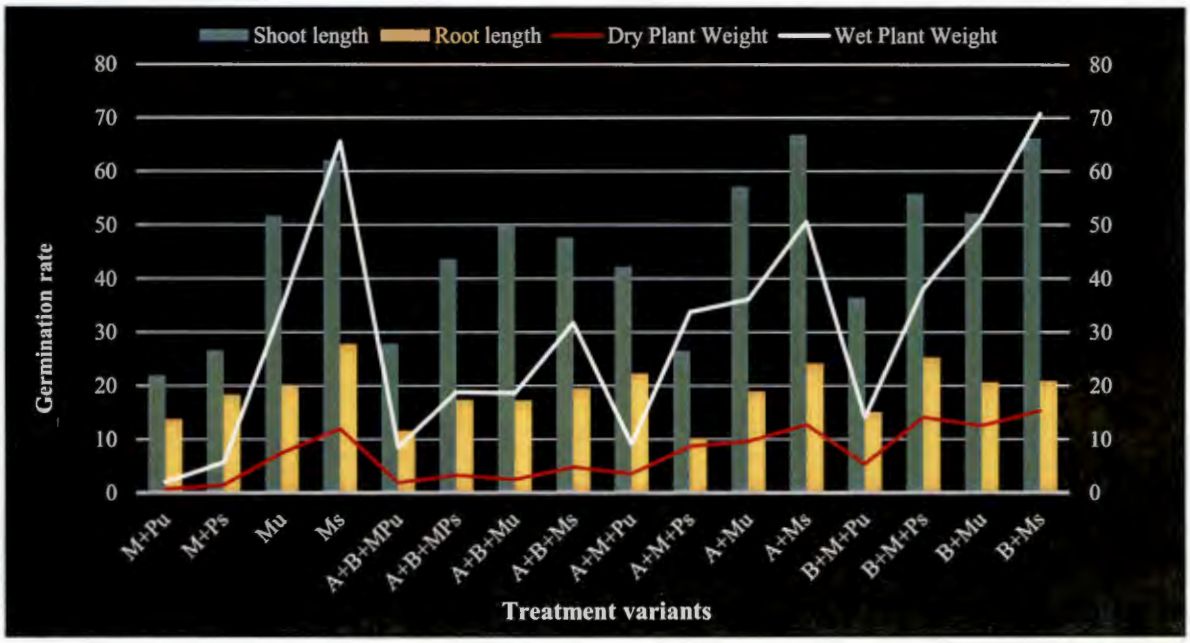


Fig. 5.4: Harvest of second experiment at V6-V7 germination stage. All values are the means of three replicated pots from two repeats. Treatments are significantly different at P = 0.05 according to Duncan's LSD test.

The harvest of the second experiment after the planting period (February 2017 – April 2017) and after the second inoculation of plants with *F. graminearum*, showed that growth was also retarded in untreated maize seedlings. The controls inoculated only with Fg had lower germination levels. The result also showed that treatments in sterilized soil without pathogen showed a higher increase in plant vigour than plants in unsterilized soil, except for the treatment with bacterial mix where pathogen was excluded (A+B+Mu and A+M+Pu). This might be because the antagonists were not exposed to competition. Furthermore, antagonist PS9.1 (A+Mu) increased plant germination when the pathogen was excluded compared to BS10.5 (B+Mu). Antagonist BS10.5 however performed significantly better than PS9.1 in unsterilized soil when co-inoculated with the fungal pathogen

(B+M+Ps/A+M+Ps; B+M+Pu/A+M+Pu). The reduced growth seen in plants with bacteria mix treatments could be due to lack of coexistence between the two bacteria, as we mentioned earlier. We did not apply any additional/external fertilization or fungicide treatment during the planting periods, to ascertain that the treatments were the only source of nutrients received by the plants during their germination period. Management of grain fusariosis remains unalleviated and it might be necessary to integrate multiple plant disease approaches including efficient cultural practices, use of resistant cultivars, fertilization or addition of low concentration of fungicide or both along with the bioprotectants to see how efficient they would be. A combination of several disease management practices has been the major approach to managing the continued incidence of cereal grain fusariosis (Aiyaz et al., 2015; Wegulo et al., 2011; Wegulo et al., 2015). The results collected agree with previous studies seen under greenhouse and field trials in which species within the genera *Pseudomonas* and *Bacillus* caused a reduction in *Fusarium* pathogens growth and aggression in cereal cultivars (Khan et al., 2001; Khan et al., 2004; Palazzini et al, 2007; Pereira et al., 2009; Pereira et al., 2011; Schisler et al., 2004; Khabbaz et al., 2015).

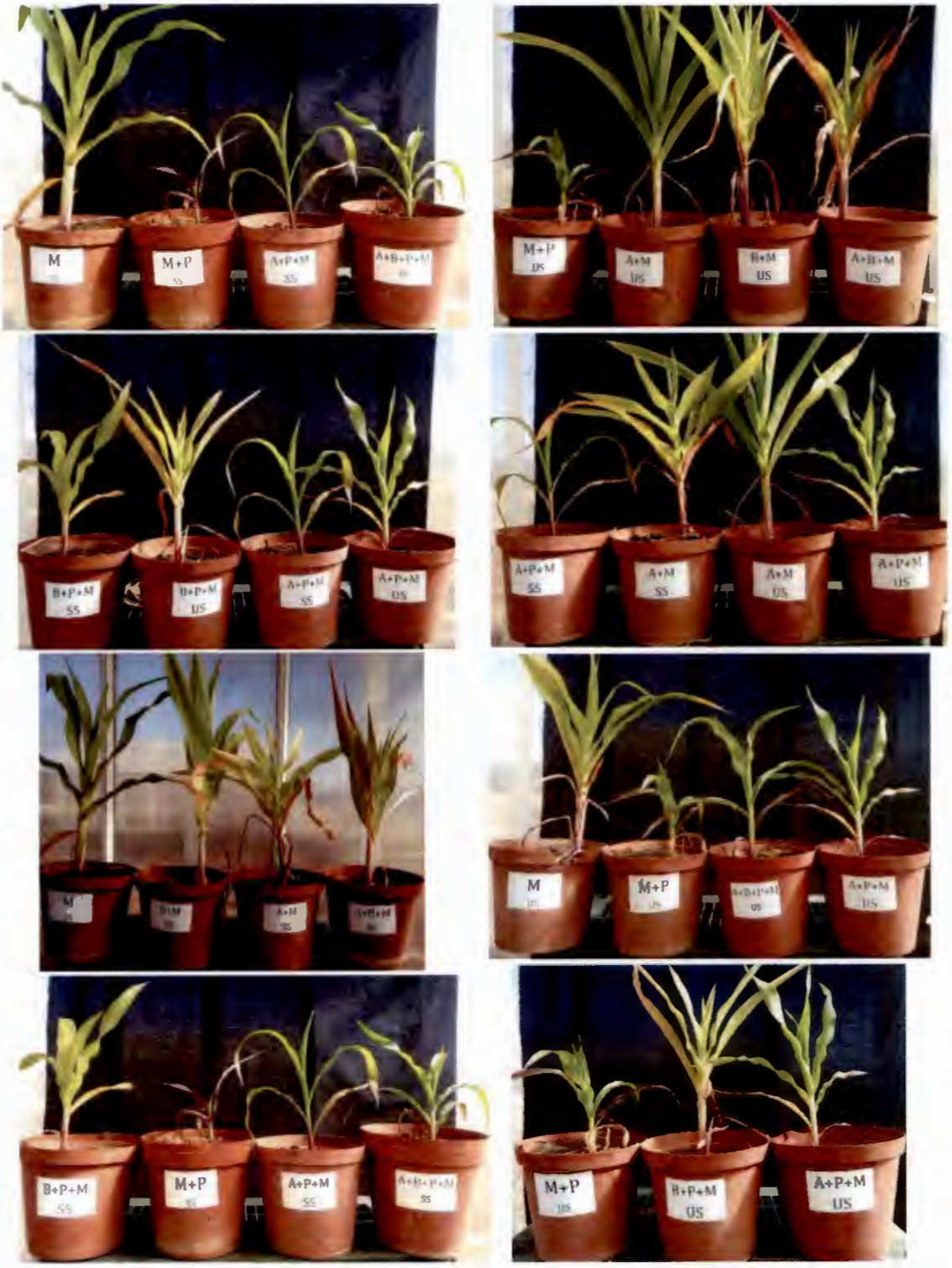


Photo 5.6: Harvest of second experiment at V6-V7 germination stage.

During the third experimental period, we increased the inoculum concentration of *F. graminearum* from 10^7spores ml^{-1} to 10^5 spores ml^{-1} to see if the incidence of fusariosis would be more evident. Plants were harvested at tasseling stage (VT). Good plant growth was recorded for seeds without treatments (Ms and Mu), however, plants with only Fg treatments (without any bacterization) dried and died off after the additional Fg treatment at increased inoculum concentration three weeks after seeding. Hence, growth parameters and vigor (shoot length, root length, fresh shoot weight, dry root weight, fresh root weight and dry root weight) were recorded for the plants that survived. **Table 5.4** shows the biosuppressive potential of the bacterial antagonists BS10.5 and PS9.1 against Fg fusariosis incidence.

Bacterial inoculations without pathogen treatments and control were not evaluated for disease incidence, however the bacterial treatments remarkably enhanced plant growth compared to the uninoculated control. In the treatments with pathogen addition, the evidence of disease suppression was significantly obvious, because there were improvements in plant growth parameters in comparison to Fg inoculated plants, which wilted and died off (see Photo 5.6). This result correlates with the report of Pandey et al. (2001). The observable survival of the bacterized maize plants is not unexpected since they are no longer distressed, and they possess a non-diseased root system as shown in Photo 5.7. Treatments with isolate BS10.5 and Fg performed better in sterilized soil compared to PS9.1 (A+P+Mu) and bacterial mix (AB+P+Mu). Also treatments with PS9.1 had better root systems which was seen throughout all the pot experiments. We observed some discolorations in the tassels from the plants harvested from sterilized soils. Also, tassels from BS10.5 treated plants were significantly larger than the control and other treatments (Photo 5.8). In this third experiment, the observed bioprotection against Fg aggression may be attributed to the

antibiotic secreting potential of the isolates, or induction of systemic resistance in maize by the isolates (Nunes and Dean 2012; Otieno et al., 2015). This might be part of further a study to identify responses elicited by maize as a result of introducing these antagonists.

Table 5.5: Effect of bacterial treatments on seed-borne incidence of *F. graminearum* on maize: harvest of third pot experiment

Treatment	Shoot Length (cm)	Root Length (cm)	Fresh Shoot Weight (g)	Fresh Root Weight (g)	Dry Shoot Weight (g)	Dry Root Weight (g)
B+P+Ms	41.08hi	17.30ef	17.28g	12.22f	4.39g	1.75fg
B+P+Mu	35.74i	14.41f	16.05g	11.59f	4.38g	1.57g
A+P+Ms	45.09gh	18.33ef	29.60e	15.05e	8.92f	2.77ef
A+P+Mu	51.72ef	21.79de	36.35d	18.50cd	11.25d	4.14d
AB+P+Ms	44.42gh	17.86ef	24.57f	15.02e	8.66f	2.50efg
AB+P+Mu	50.01fg	19.10e	31.84e	16.26de	9.12ef	2.99e
AB+Ms	50.78efg	21.74de	36.31d	16.51de	10.44de	4.13d
AB+Mu	56.63de	24.84cd	39.45cd	19.02bc	11.39d	4.92cd
Ms	61.12d	24.98cd	39.64cd	19.32bc	11.55d	4.99cd
Mu	69.13c	26.21cd	40.45cd	20.08bc	14.23c	5.08cd
B+Mu	72.76bc	26.24cd	42.21bc	20.30bc	15.27bc	5.28c
B+Ms	78.75ab	32.63b	52.67a	21.52ab	18.21a	6.42b
A+Mu	74.16bc	29.24bc	45.23b	21.32ab	16.11b	6.31b
A+Ms	83.13a	38.52a	53.79a	22.83a	18.31a	7.41a
Mean	58.18	23.80	36.10	17.82	11.59	4.30
SE	0.56	0.38	0.37	0.22	0.13	0.09

Harvest of third experimental period. Reduction of incidence of fusariosis at 90days after artificial pathogen inoculation. All values are the means of four replicated pots. Treatments are significantly different at P = 0.05 according to Duncan's LSD test. Values with same letters are not significantly different. SE: Standard error.

Photo 5.7: Fg aggression observed in the non bacte0rized maize seedling germination.

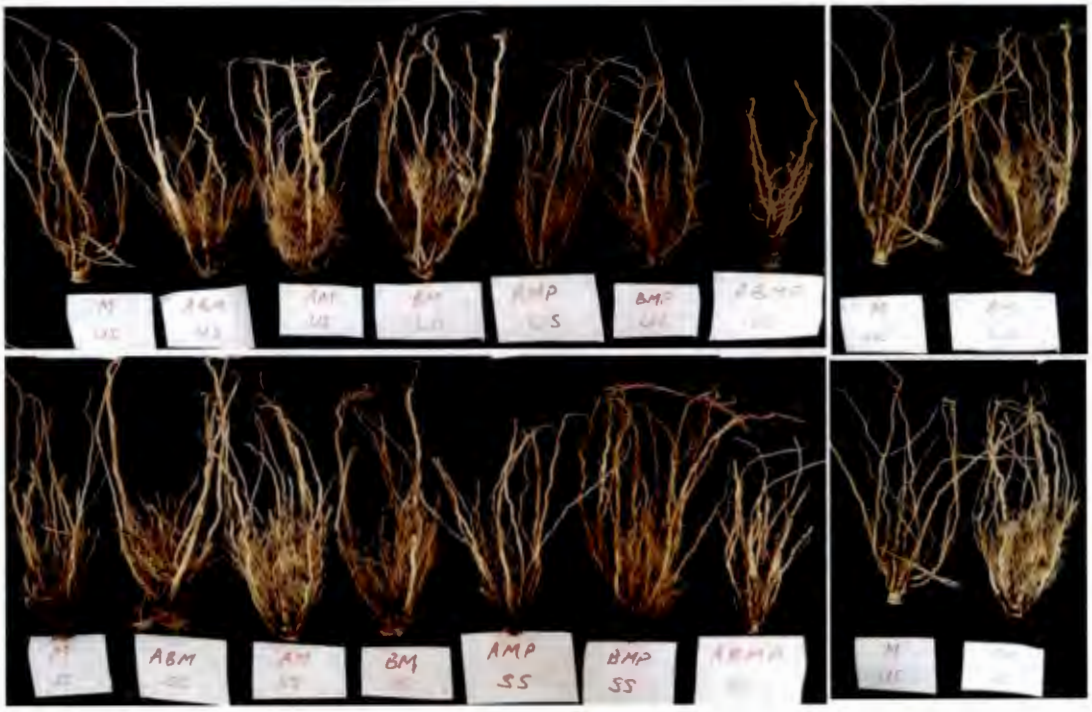


Photo 5.8: Bioprotective effects of antagonist on root system development

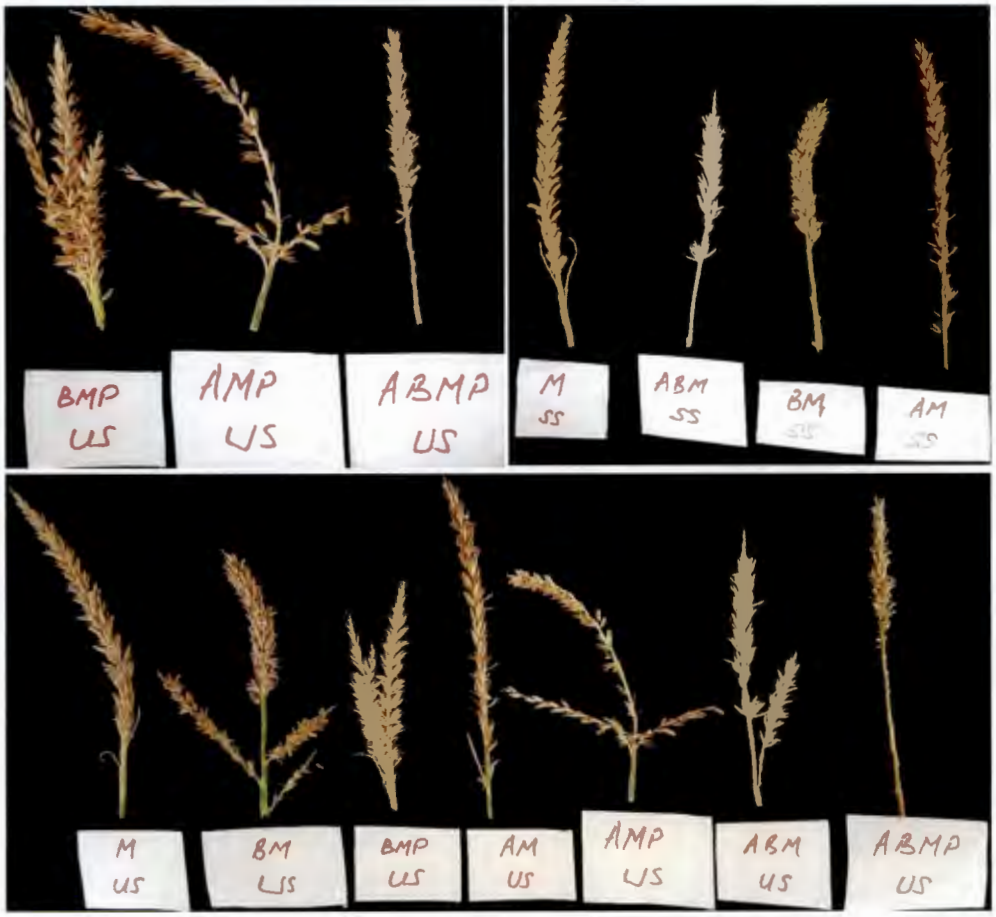


Photo 5.9: Bioprotective effects of antagonist seen on tassel development

Biocontrol inoculant formulations and their mode of delivery are important for their successful under field conditions (Babalola, 2010). From the result we gathered, the root dip treatment of maize seedling appears to be effective in conferring bioprotective ability on the maize. This correlates with the reports of Pal et al. (2001) and Pereira et al. (2010), in which pre-sowing of seedlings enhanced the activities of BCAs. The root dip approach ensures that the microbial

agents do not just actively colonize the surface of the host but possibly also become endophytic. This can lead to the activation of the plant's defense system (Cook et al., 1996).

Conclusion

The significant findings of the study are the antibacterial potential of the PS9.1 and BS10.5, their reduction of maize seedling fusariosis and the effectiveness of the rhizobacterial antagonists in unsterilized soil. This gives a hint of their ability to persist and compete in the environment against resident microflora if they are considered as biocontrol candidates. In as much has maize germination was protected against Fg aggression, further work to determine the reduction of mycotoxin contamination or level of nutrient uptake by treated seeds might be necessary to completely ascertain the full bioprotective potential of these rhizobacterial strains.

CHAPTER SIX

GENOME SEQUENCE OF *BACILLUS VELEZENSIS* NWUMFK_BS10.5, A PROMISING BIOCONTROLLER FOR MAIZE (*ZEA MAYS. L*) FUSARIOSIS

Abstract

Genomes of members of the gram-positive bacterium *Bacillus velezensis* have been sequenced and several strains have been used as plant growth promoters and biocontrollers. We isolated strain BS10.5 which harbors biosynthetic genes for the production of known lipopeptides such as surfactin, fengycin, plipastatin and iturin. Major clusters for the synthesis of antibiotics and other important secondary metabolites were identified in this candidate biocontrol strain.

6.1 Introduction

Fusariosis of *Fusarium graminearum*, a world-wide cereal phytopathogen, has been adversely affecting small grain yield and quality (Wegulo et al., 2015). The traditional plant disease management practices often utilized for the control of fusariosis, such as application of chemical fungicides and crop resistant cultivars, have been less effective in alleviating the situation in South Africa (Boutigny et al., 2011, 2012). To provide an effective, environmentally friendly alternative approach for Fusariosis management, we sought for a biocontrol strain having survival traits peculiar to the South African maize cultivation conditions. We isolated and identified based on 16s rRNA gene an indigenous *Bacillus sp.* BS10.5 (accession no. KX353617) from the maize rhizosphere that appears to exhibit strong potential for *F. graminearum* suppression *in vitro* (unpublished data). In recent years, genomic information of *Bacillus velezensis* and other related *Bacillus* strains having strong potential for the control of phytopathogens including *F.*

graminearum have been made available (Lee et al., 2015; Palazzini et al., 2016; Pan et al., 2017), however there has been recent taxonomic reclassification of *B. velezensis* sp. (Dunlap et al., 2016 and Fan et al., 2017). In the light of this, we sequenced the genome of BS10.5 to further explore its genomic potential due to its unique antimicrobial traits.

6.2 Methods and Results

The whole genome of the *Bacillus* strain BS10.5 was sequenced with Illumina MiSeq reagent kit v2 micro system. The sequencing yielded a total of 7,505,117 clusters and 15,010,234 paired-end reads with an average read length of 151 bp. The Kbase (Arkin et al., 2016) platform was used to check quality of reads (FastQC v.1.0.1), trim reads (Trimmomatic v0.32) close gaps and remove adaptor sequences (Cutadapt v1.0.1). The reads were also assembled into contigs on the Kbase platform using ARAST v0.0.4 (Velvet and Kb_SPAdes v0.0.9). Contigs from the Kbase were uploaded on the RAST Server version (v) 2.0 (Aziz et al., 2008), PATRICK v3.3.15 (Wattman et al., 2017) and NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP v4.2) (Pruitt et al., 2012) for automated annotation. After compilation of the data, the genome yielded between 3,905,592 and 3,964,473 bp, and G+C content of 46.4% Table 6.1. Subsystem information of the isolate was predicted in SEED viewer (Overbeek et al., 2014) (Figure 6.1) and the genome was blast compared with closely related *Bacillus* strains using GViewer server (Figure 6.2). Isolate BS10.5 was finally identified as a *B. velezensis* strain.

Analysis of the secondary metabolite biosynthesis gene clusters with antiSMASH v4.0.0rc1 (Weber et al., 2015) revealed that BS10.5 had 16 gene clusters involved in secondary metabolic activities. An overview showed that the clusters were devoted to the synthesis of

antimicrobial peptides such as surfactin, mersacidin, fengycin and oocydin. The non-pathogenicity of BS10.5 was determined by Pathogenfinder v1.1 web server (Cosentino et al., 2013).

6.3 Conclusion

The vast antimicrobials identified in BS10.5 genome makes it a promising strain for biocontrol research (Ongena et al., 2008). BS10.5 is been utilized for preliminary trials for *F. graminearum* biocontrol, therefore this whole genome information will provide deeper understanding on the mechanism by which it suppresses *F. graminearum* and it should provide possible ways of bioengineering the strain.

6.4 Strain and nucleotide sequence accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NZ_NITU01000037.1. The version described in this chapter is version NZ_NITU01000037.1. The BioProject and Biosample designation for this project is PRJNA388288 and SAMN07174738. The datasets generated during and/or analysed during the current study are available on reasonable request.

Table 6.1. Genome attributes of *B. velezensis* NWUMFk_BS10.5 compared with other *Bacillus* spp. in the *Bacillus velezensis* group.

Attributes	*B. velezensis* BS10.5	*B. velezensis* FZB42	*B. velezensis* AS43.3	*B. velezensis* UCMB5113
Size of genome (bp)	3,964,473	3,918,589	3,961,291	3,889,532
Number of subsystems	370	-	-	-
G + C numbers (%)	46.39	46.49%	46.60%	46.71%
Number of coding sequences	3875	3693	3861	3656
Total genes	3916	3421	4037	n.a*
tRNA	89	89	89	89
rRNA	13	9	29	10
Number of RNAs	93	117	118	182

n.a*: not applicable

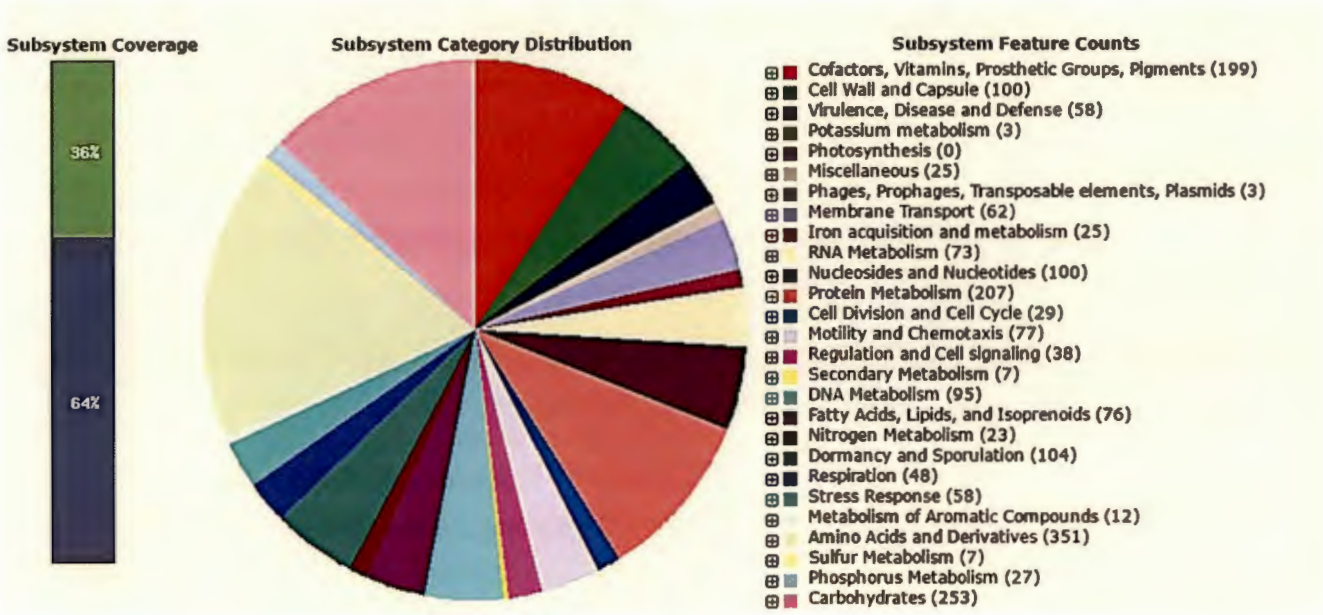


Fig. 6.1: Subsystem summary of the genome *Bacillus velezensis* NWUMFk_BS10.5 predicted by SEED Viewer v2.0. Genomic features are colored according to their functional classification types (Overbeek et al., 2014).

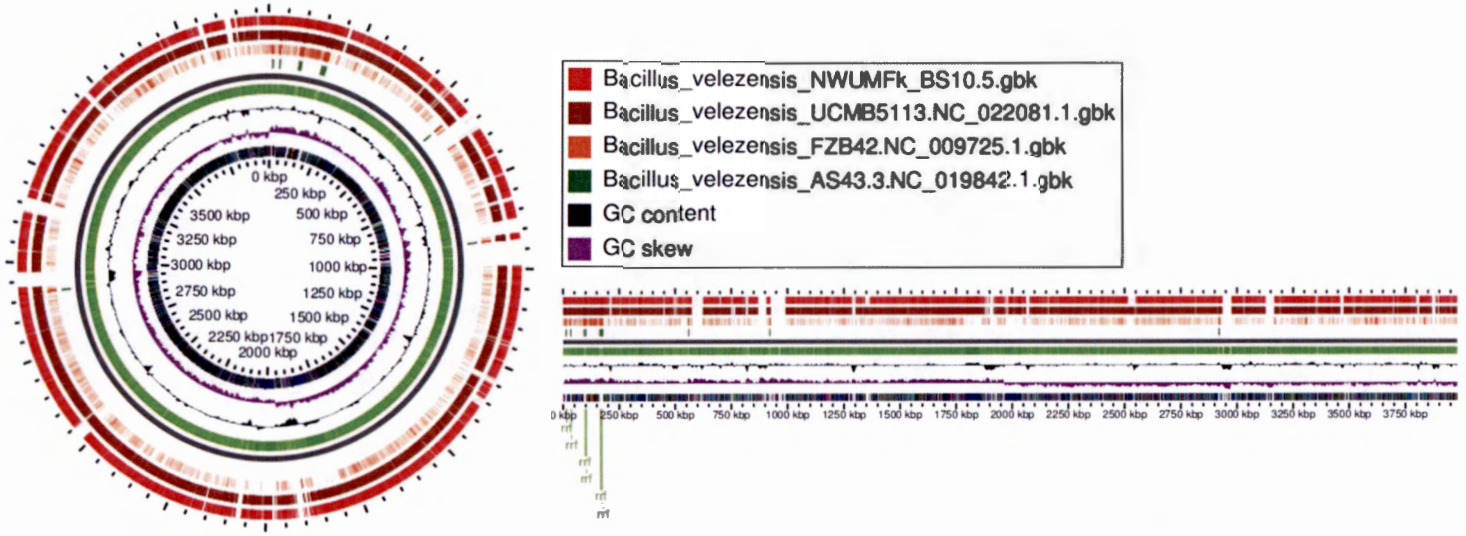


Fig. 6.2: Blast atlas of NWUMFk BS10.5 and closely related *Bacillus velezensis* strains. Ribosome recycling factor is shown on the linear map as *rrf*. Genome features compared include rRNA, tRNA, genes and coding sequences (CDS). Circular map was generated by GView Server (GPLv3) (Petkau et al., 2010).

CHAPTER SEVEN

CHARACTERIZATION OF THE LIPOPEPTIDE SUBSTANCE AND GENOMIC MINING OF *BACILLUS VELEZENSIS* BS10.5

Abstract

Bacillus sp. BS10.5 was selected from among several antagonists isolated from the maize rhizosphere because it exhibited strong and consistent antimicrobial activity against the phytopathogens *F. graminearum*, *F. culmorum* and also against well-known bacterial pathogens. The logarithmic phase compounds secreted by the isolate also showed remarkable antimicrobial potentials and they were further extracted. Structural elucidation by NMR, FTIR and mass spectrometric analysis of the extracts by ESI-Q-TOF MS, confirmed the presence of the compounds iturin, fengycin and surfactin in the extracts of *Bacillus* BS10.5. These compounds are identified to have played a key role in the biocontrol potential of PS9.1 and BS10.5. Mining the genome sequence data of isolate BS10.5 revealed the locations of putative functional antibiotics, other secondary metabolites and possibly unidentified biosynthetic genes present in its core genome. Lastly, the genomic sequencing of BS10.5 also revealed its robust capacity for other beneficial biotechnological applications.

7.1 Introduction

Microorganisms secrete several important metabolites during their growth phases and some of their metabolic activities during the logarithmic phase produce compounds that make them important to man. These beneficial organisms are sometimes utilized in crop management practices to improve plant growth or control pathogens. The mechanisms employed by biocontrol agents and the chemical basis of these biocontrol properties have been well studied over the years (Raaijmakers et al., 2002; Lugtenberg and Kamilova, 2009; Santoyo et al., 2012; Goswami et al., 2016). Among the various bacterial genera that have been established to exhibit biocontrol potential is the genus *Bacillus*. Several members of the genus have been identified to possess biosynthetic capability to produce a wide range of genetically encoded antimicrobial peptides (AMPS) that are promising alternatives to artificially synthesized antimicrobial compounds and these AMPS are either ribosomally or nonribosomally synthesized (Ongena and Jacques 2008; Sumi et al., 2014; Cochrane et al., 2016).

Strains within the genus have also been reported to synthesize structurally diverse secondary metabolites that exhibit broad spectrum antibiotic activities and the genomic basis for the synthesis of these secondary metabolites has been attributed to the presence of polyketide synthases (PKSs) and nonribosomal peptide synthetase (NRPS) in their genomes (Stein 2005; Raaijmakers et al., 2006; Roongsawang et al., 2011; Tyc et al., 2017). The amphipathic structure, the hydrophilic peptide portion and a hydrophobic fatty acid portion of these peptides show similar resemblance. These peptides also exhibit a cyclic nature due to the linkage of their C-terminal peptide residue either indirectly to a β-hydroxy fatty acid or directly to a β-amino acid (Ongena and Jacues 2008 and Mnif et al., 2015). These antimicrobial peptides have been isolated, quantified, purified and characterized using various approaches and techniques that ensure the

chemical components responsible for their bioactivity are well understood. The majority of the current approach employed involves the combination of chromatographic techniques, mass spectrometry, Nuclear Magnetic resonance (NMR) and Fourier Transform Infrared spectroscopy (FTIR) (Deepika et al., 2015; Biniarz et al., 2016; Jasim et al., 2016).

Reports have shown that expression of biosynthetic genes and secretion of secondary metabolites may be difficult during laboratory culture of potential BCA due to growth conditions (Laureti et al., 2011). The lack of expression of genes or secretion of secondary metabolite can hinder the identification or detection of the specific metabolite or gene responsible for the antimicrobial activities of a BCA (Michelsen et al., 2015). To fully understand beneficial bacterial species, genomes of multiple independent isolates are required for comparison (Tettelin et al., 2005). Comparing the total repertoire of genes for a group of genomes from close bacterial species is an instrumental approach for the development of novel beneficial compounds (e.g medical vaccines) and for the functional characterization of important genetic determinants in significant microbial strains (Medini et al., 2005). The bacterial pan-genome, can be defined to be the complete repository of genes located in the genome of closely related bacterial species. This includes the 'core genome' (genes identified in two or more strains) and the 'dispensable genome' (genes peculiar to single strains) (Tettlin et al., 2005; Medini et al., 2005; Rouli et al., 2015). The core and dispensable genes are crucial signatures for recognizing species diversity.

Researchers now tend to use a combinational approach such as genome mining, pan-genome analysis, structural data elucidation and chemical characterization to identify biosynthetic products (Schulze et al., 2015) secreted by important microbes. Often, genomic data offers predictions that lead to the detection of novel biosynthetic pathways, genes and enzymes which then enables experimental isolation, structural elucidation and chemical characterization of novel

compounds (Challis et al., 2008). The combinational approach ensures that *in silico* or theoretically predicted biosynthetic products correlate with structurally or chemically identified metabolites (Ziemert et al., 2016). Here we examined the antimicrobial potential of the lyophilized extract of the secondary metabolites secreted by *Bacillus* sp. BS10.5 while employing Electro Spray Ionization Mass Spectrometry (ESI-Q-TOF MS), FTIR and NMR to understand the functional and structural components of the compounds extracted. We further profiled the whole genome of BS10.5 which was previously sequenced (Chapter Six) with the intent of identifying its internal components and exploring its genomic capability. We should gain deeper insights that can help us better utilize this *Bacillus* strain from the genome information.

7.2 Materials and Method

7.2.1 Extraction, collection of cell free supernatant and purification of secondary metabolites

The production and purification of the active metabolites were done according to Gond et al., (2015) with slight modification. Cell free antimicrobial substances from BS10.5 were collected after the rhizobacteria grown in 1 L LB-broth at 30 °C with continuous shaking at 200 rpm for 72 hr. The cells were harvested by centrifugation at 13,000 g for 15 min, and the culture supernatant was filter sterilized through 0.22 µm nitrocellulose membranes (Millipore Corporation, Bedford, MA, U.S.A.) filters to obtain cell free supernatants. 100 ml of cell free supernatant was stored for anti-pathogen test.

Isolate BS10.5 was further grown in 1 L LB-broth at 30 °C with constant shaking at 200 rpm for 4 days. After fermentation, the cell filtrate was collected by centrifugation at 6000 rpm for 15 min at 4°C and the supernatant was acid precipitated by adjusting to pH 2.0 with 6 M HCl.

After an overnight incubation at 4°C, the precipitate was centrifuged at 8000 rpm at 4°C for 15 min and the pellet was dissolved in methanol-water (50:50) and then filtered through 0.22 μm PTFE membrane filter to remove larger particles and cell components. The mixture was further concentrated by vacuum evaporator at 45°C and then finally lyophilized.

7.2.2 Effect of culture free supernatant and lyophilized extracts of BS10.5 on bacterial pathogens and *Fusarium* pathogens

7.2.2.1 Antibacterial activity

Activity of the cell free supernatants was determined by disc diffusion assay. Sterile filter paper discs were impregnated with 60 μl of the cell free supernatant of BS10.5 and the disc were placed on the MHA plate previously inoculated with bacterial pathogens (BC=*Bacillus cereus* ATCC 10876, EF=*Enterococcus faecalis* ATCC 29212, KP=*Klebsiella pneumoniae* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853). Control plates included antibiotic discs of Norfloxacin (5 μg/disc) and Tetracycline (30 μg/disc) and the plates were incubated overnight at 25°C and 28°C. Antibacterial activity was observed as inhibition zones around the disc and the experiment were conducted twice in triplicates.

7.2.2.2 Antifungal activity

Sterile filter paper discs impregnated with 60 μl of the cell free supernatant of BS10.5 were placed at the far edge of a PDA plate and 5 mm agar plug of *Fusarium* pathogens (*F. graminearum* and *F. culmorum*) were transferred to the opposite edge of the PDA plates. Nystatin (30 μg/disc) was used as control and after 7 days of incubation and observation of plates at 25°C, zones of inhibition were recorded.

7.2.3 Determination of the antimicrobial activity of lyophilized extract of *Bacillus* BS10.5

7.2.3.1 Anti-pathogenic activity of the lyophilized extract in the presence of different diluents

Anti-pathogenic activity of the lyophilized extract dissolved in several diluents/solvents was compared. Previous reports showed that antimicrobial activity of extracts or compounds dissolved in solvents was also enhanced by the solvents. 0.1g portion of the lyophilized extract was dissolved in 1000 µl of butanol, DMSO, Ethyl acetate, Chloroform, PBS and Benzene and 60 µl portion of each mixture was then loaded into each of the wells (made previously with bottom parts of 200 µl sterile pipette tips) of MHA plate previously inoculated with bacterial pathogens (KP, PA, BC and EF) or 5 mm agar plug of fungal pathogens (*F. graminearum* and *F. culmorum*). Wells loaded with each solvent (without lyophilized extracts) were used as a control in both agar plates. As a result each plate (MHA or PDA) had six discs per pathogen.

7.2.3.2 Anti-pathogenic activity of the lyophilized extract at different concentrations using PBS as diluent

From the previous results above, only PBS had no direct impact on the activity of the lyophilized extract powder when used as diluent. So we further determined the anti-pathogenic activity of the extract powder at different concentrations using PBS as diluents. Disc diffusion was employed for the antimicrobial assay following the method described by Chen et al., (2010) with modifications. The lyophilized extract was dissolved in phosphate buffered saline (PBS) (pH 7.5) to a concentration of 10 mg/ml and serially diluted to varying concentrations of 100, 90, 70, 50, 30 µg/ml. Sterile discs made from punctured filter papers were then impregnated with 40 µl of the solutions and allowed to dry. Impregnated discs were then placed on the periphery of a freshly prepared PDA plate containing 5 mm disk of the fungal pathogens (*F. graminearum* and *F. culmorum*) in the center or MHA plate in which 100 µl of overnight cultures of the bacterial

pathogens at OD 0.5:600 nm had been spread. Forty microliters of PBS (pH 7.5) was used as control disc. Antimicrobial activity was observed by the inhibition of microbial growth around the disk. The plates were incubated for 5 days at 28°C for fungal pathogens and 2 days at 28°C for bacterial pathogens (KP, PA, MC and EF). Each test consisted of 3 replicates. Recordings were taken if zones of inhibition were spotted from the border of the disc to the perimeter of visible pathogen.

7.2.4 Effect of lyophilized extracts and commercial fungicide on *F. graminearum* and *F. culmorum* growth

7.2.4.1 Anti-pathogenic activity of the lyophilized extract and commercial fungicides on fungal mycelia

Using well diffusion, the activity of the BS10.5 extract powder was also compared to the activity of commercial fungicides using PBS as diluent following a modified protocol of Mousa et al. (2015). 0.2 g/ml of the lyophilized extract, 10 µg/ml of amphotericin B, 10 µg/ml of Nystatin and triazole (10 µg/ml) were prepared with PBS (pH 7.5). Each mixture was then loaded into each of the wells (made previously with bottom parts of 200 µl sterile pipette tips) of PDA plates previously inoculated with 5 mm agar plug of fungal pathogens (*F. graminearum* and *F. culmorum*). The plates were incubated for 5 days at 28°C and each plate consisted of 3 replicates. Recordings were taken if zones of inhibition were spotted from the border of the well to the perimeter of visible pathogen.

7.2.5 Identification and characterization of bioactive compounds by NMR, FTIR and ESI-QTOF-MS analysis

7.2.5.1 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is a technique employed to identify the types of chemical bonds and functional groups in compounds and it is useful in elucidating the components of an unknown sample. 10 mg of the lyophilized extract of BS10.5 sample collected from day 1-4 of fermentation was analyzed with Transform Alpha (FT-IR) KBr integrated spectrometer (Bruker). Spectrum was collected at 400 to 4000 wavenumbers (cm^{-1}) with an average of 32 scans. The spectrum was viewed and collated with the OPUS spectroscopy software.

7.2.5.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is used to determine the physical and chemical properties of molecules. 20 mg of sample dissolved in 0.5 ml of deuterated DMSO and ^{1}H and ^{13}C NMR spectrum were acquired on the specific signal assignment. NMR spectra were recorded using a Bruker AvanceIII 500 MHz spectrometer at room temperature with chemical shifts (δ) recorded against the internal standard, tetramethylsilane (TMS).

7.2.5.3 Mass Spectrometry Analysis by ESI-QTOF-MS

To determine the molecular mass of the purified substance accurately, a Micromass ToFSpec matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MICRO-TOF-MS) was carried out. A high-resolution mass spectrum was obtained for the purified sample with an Applied Biosystems 4800 Plus MICRO-TOF/TOF analyzer (AB Sciex, USA) operated in the positive ion mode with an accelerating voltage of 20 kV, 337 nm nitrogen laser for ionization, and a-cyano-4-hydroxycinnamic acid for matrix. Bruker compass data analysis was used to process

the mass spectrometry data while molecular weights and formulae were characterized by mass spectrum smart formula tools.

7.2.6 Data mining and *in-silico* bioinformatic analysis of the BS10.5 genome

Sequencing of BS10.5 genome was carried out earlier by Ilumina (Chapter Six) and antibiotics and secondary metabolites were predicted by Anti-smash version 4.0.0rc1. Here we further explore the genome *in silico* based on the Anti-smash and PRISM data with the aim of describing the biosynthetic products or genetic clusters present in BS10.5. A Pangenome was created on the Kbase platform to run a comparison of the total genes present in BS10.5 and other established PGP from the *Bacillus* spp. In addition, the combination of Kbase and SEED viewer/KEGG platforms was used in building a metabolic model for strain BS10.5 from its annotated genome.

7.2.7 Statistical analysis

A Multivariate General linear model was used to analyze data (treatment means, and inhibition rates). Least significant difference test (LSD), Duncan multiple test and Student-Newman-Keuls (SNK) test were used to compare observed means, pathogen-antagonist relationship, treatment effects and effect of conditions of inoculation using SPSS statistical package programme (version 22) at the significance level of 5%.

7.3 Results

7.3.1 Antimicrobial activity of cell free supernatants

The results of the antimicrobial activity of the cell free supernatants showed that cell-free culture filtrate from BS10.5 contains strong bioactive substances with inhibitory potentials against fungal and bacterial pathogens Table 7.1 and Photo 7.1. The cell free filtrates inhibited Fg and *B. cereus* at same rate based on our scale of assessment, but only showed moderate inhibition of *K. pneumoniae* and *P. aeruginosa*. In comparison with antibiotics used, the cell free supernatant exhibited close inhibition level with nystatin against Fcul and Fg. However, it showed better inhibitory potential than tetracycline and ciprofloxacin against the bacterial pathogens based on the concentration of the antibiotics used in this assay.

Table 7.1: Inhibition rates of cell free supernatants of BS10.5 on microbial pathogens

	Pathogens					
Test substances	Fcul	Fg	KP	PA	EF	BC
BS10.5	+++	++++	++	++	+++	++++
Ciprofloxacin	ND	ND	+	+++	++	++
Tetracycline	ND	ND	+++	-	++	++
Nystatin	+++	+++	ND	ND	ND	ND

Fcul = *F. culmorum*; Fg = *F. graminearum*; KP = *Klebsiella pneumonia* ATCC 25923, PA = *Pseudomonas aeruginosa* ATCC 27853 EF = *Enterococcus faecalis* ATCC 29212, BC = *Bacillus cereus* ATCC 10876. + = **zone of inhibition**, - = no inhibition zone: (+ = weak; ++ = moderate; +++ = good; ++++ = high, represent relative inhibition rates of supernatants on growth of each pathogen on the Lb-PDA agar to the level of 10-29%, 30–49%, 50–69% and ≥70%, respectively.

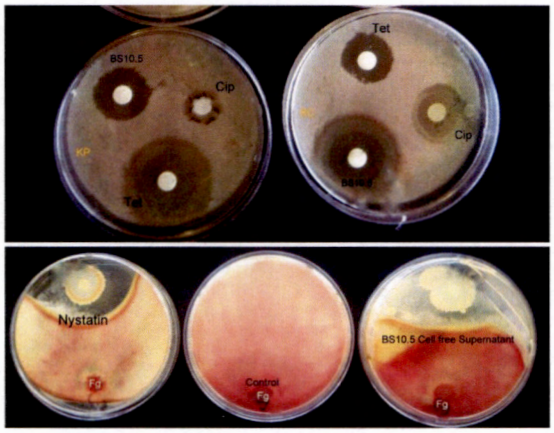


Photo 7.1: Inhibition rates of cell free supernatants of BS10.5 on microbial pathogens. Effect of tetracycline, BS10.5 and ciprofloxacin on KP and BC (Upper plate); effect of nystatin and BS10.5 on Fg (lower plate).

7.3.2 Anti-pathogenic activity of the lyophilized extract in the presence of different solvents

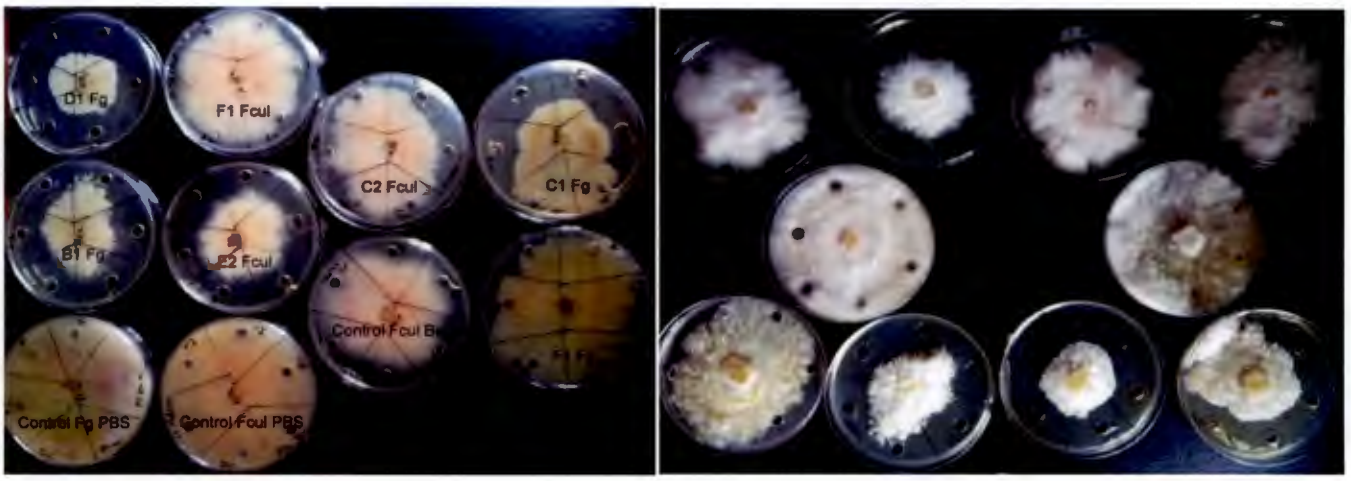
Our evaluation of the effects of solvent on antagonistic activity of crude microbial extracts shows that the solvents positively enhance their activity (Table 7.2). Only the extract dissolved in PBS showed stable activity, because the PBS control had no inhibitory zone on the test bacteria and activity of the extract was not enhanced by PBS.

Table 7.2: Antimicrobial effects of bacteria extracts using different solvents

Extract in test solvent/diluent	Inhibition rate					
	Fg	Fcul	KP	PA	BC	EF
BS10.5 extract-Butanol	+++	++	++	++	+	++
BS10.5 extract-DMSO	+++	+++	++	++	+	+
BS10.5 extract-Ethyl acetate	+++	+++	++	++	+	++
BS10.5 extract-Chloroform	+++	+++	++	++	++	++
BS10.5 extract-PBS	++	+++	++	++	++	++
BS10.5 extract-Benzene	+	+++	++	-	-	-
Butanol*	++	+++	++	++	++	+
DMSO*	+++	+++	+++	++	++	+
Ethyl acetate*	++	++	++	++	++	+
Chloroform*	++	++	++	++	++	+
PBS*	-	-	-	-	-	-
Benzene*	+	+	+	+	+	-

Fcul = *F. culmorum*; Fg = *F. graminearum*; KP = *Klebsiella pneumonia* ATCC 25923, MC = *Moxarella cartarrhalis* ATCC 25240, PA = *Pseudomonas aeruginosa* ATCC 27853 EF = *Enterococcus faecalis* ATCC 29212, BC = *Bacillus cereus* ATCC 10876. + = **zone of inhibition**, - = no inhibition zone: (+ = very weak, 2mm-5mm; ++ = weak, 6mm–9mm; +++ = moderate 10mm–13mm; ++++ = good ≥14mm, represent relative inhibition rates of dissolved extracts on growth of each pathogen on the PDA agar respectively.

Photo 7.2: Antimicrobial effects of BS10.5 extracts on fungal pathogens using different solvents (well diffusion).



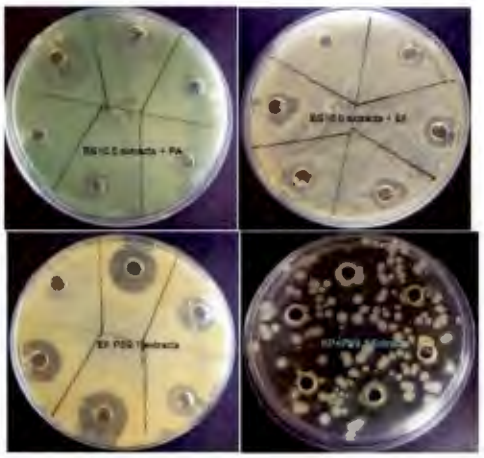


Photo 7.3: Antimicrobial effects of BS10.5 extracts on bacterial pathogens using different solvents (well diffusion).

7.3.3.1 Anti-pathogenic activity of the lyophilized extract BS10.5 at different concentrations using PBS as solvent.

During the lyophilized extract anti-pathogenic test, the effect of the extracts decreased relative to increase in dilution with PBS. The higher the concentration of the extract the higher the inhibition zones seen (Figure 7.1). Of the bacterial pathogens, EF was the most susceptible to the lipopeptides extracts of BS10.5, while MC was the least susceptible. Fg was more sensitive to the extract concentrations than Fcul. At 30 µl three of the pathogens (PA, MC and KP) showed no sensitivity.

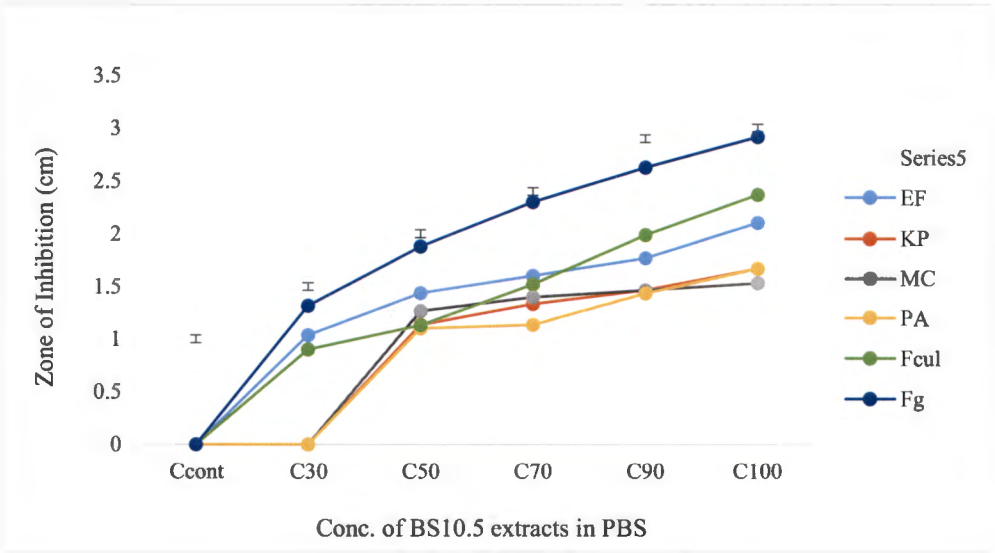


Fig. 7.1: Means of three replicates showing activity of BS10.5 extracts on the microbial pathogens at different concentrations. Values are significantly different according to Duncan's least significant difference test at $P \leq 0.05$ and means are significantly different from the control.

7.3.3.2 Anti-pathogenic activity of the lyophilized extract and commercial fungicides on fungal spores

Based on the concentrations we used for the comparative antifungal activity between the BS10.5 extract and the commercial fungicides, the highest inhibition recorded during the test was from BS10.5 crude extract followed by nystatin and the amphotericin was the most resisted by Fg (Figure 7.2 and Photo: 7.4).

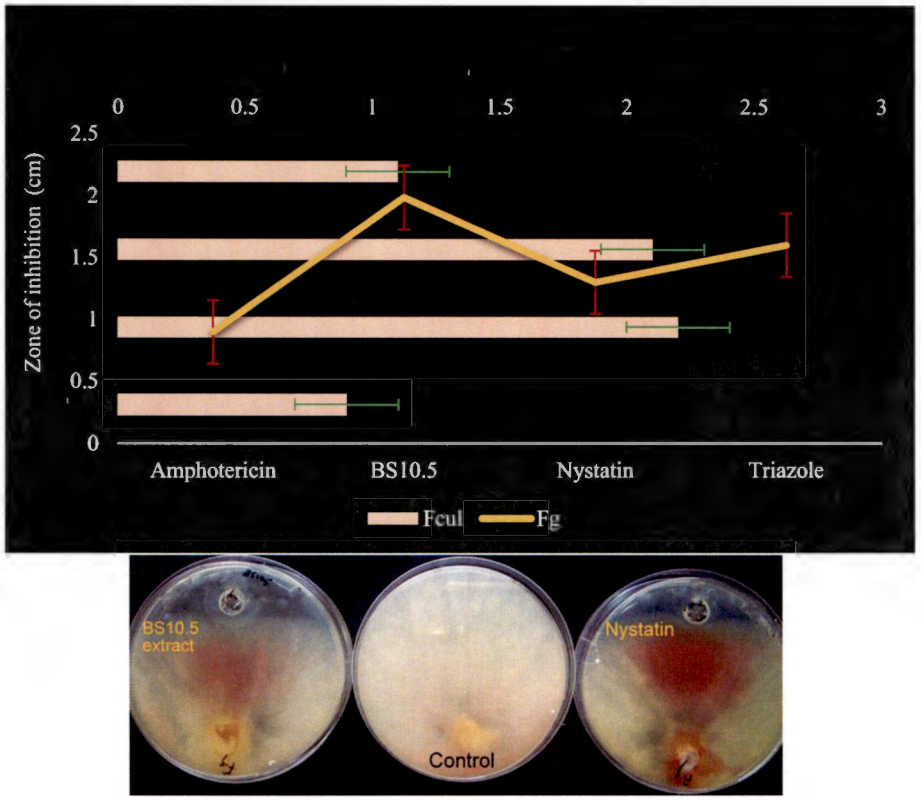


Fig. 7.2 and Photo 7.4: Inhibitory effect of the BS10.5 extract (20 mg/ml) and fungicide controls (triazole, amphotericin B and nystatin (at concentrations 10 µg/ml, respectively), on the Fg and Fcul growth *in vitro*. For the experiment, n = 4. Activity of the lyophilized extract and commercial fungicide on fungal growth were significantly different at $P \leq 0.05$.

7.3.4 FTIR, NMR and ESI-QTOF-MS analysis of the bioactive compounds present in extracts of BS10.5

7.3.4.1 Chemical analysis and structural elucidation of BS10.5 extract

From the FTIR absorbance day 1, 2, 3 showed similar peaks, but from day 4 extract, the following functional groups were observed: OH (3600-3500 cm-1), CH stretch (3000-2500 cm-1), NH stretch (2500-2000 cm-1), COO- (1900-1500 cm-1), CC and CN (1500-1000 cm-1) (Figure 7.5a and b). These wave numbers show similar characteristics of lipopeptides. The stretching and vibration mode of the absorbances is indicative of aliphatic chains, alkyl chains, peptide bonds and amide 2 bond, signifying the presence of a compound with esters and amino groups. This result, which is consistent with the report of Romero et al. (2007); Rivardo et al. (2009) and Nam et al. (2015) is indicative of fengycin, surfactin and iturin moieties.

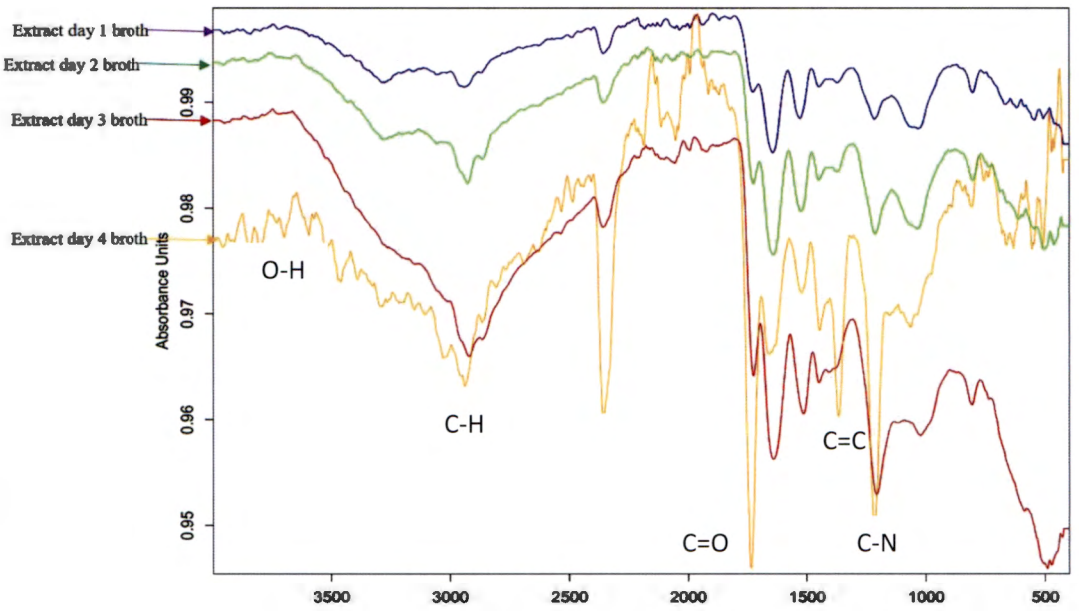


Fig. 7.5a: FTIR peaks of purified BS10.5 extract.

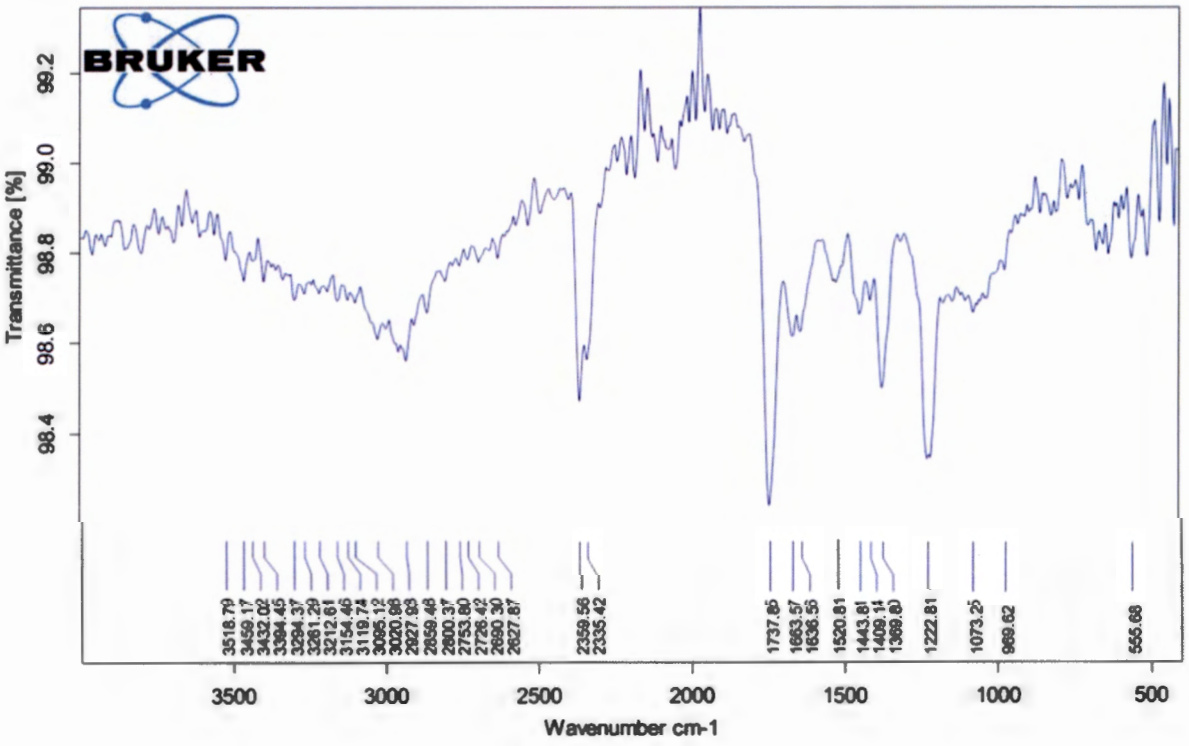


Fig. 7.5b: FTIR peaks of purified BS10.5 extract from day 4 of fermentation

7.3.4.3 NMR spectrum

The proton analysis of the compound showed NH and OH proton at >8.00 ppm, some – CH_3 and CH_2 signals at <2.00 ppm and CH_2-COO > 2.00 ppm but < 4.00 ppm. These are suggestive of a peptide backbone of secondary amide, aliphatic chains and ester linkages respectively. Chakraborty et al., (2014), gave a similar report from the proton spectra of the lipopeptide extract of *B. vallismortis* JB201 and *B. subtilis* SJ301. The signals of the carbon analysis was inconclusive despite several runs (see appendix).

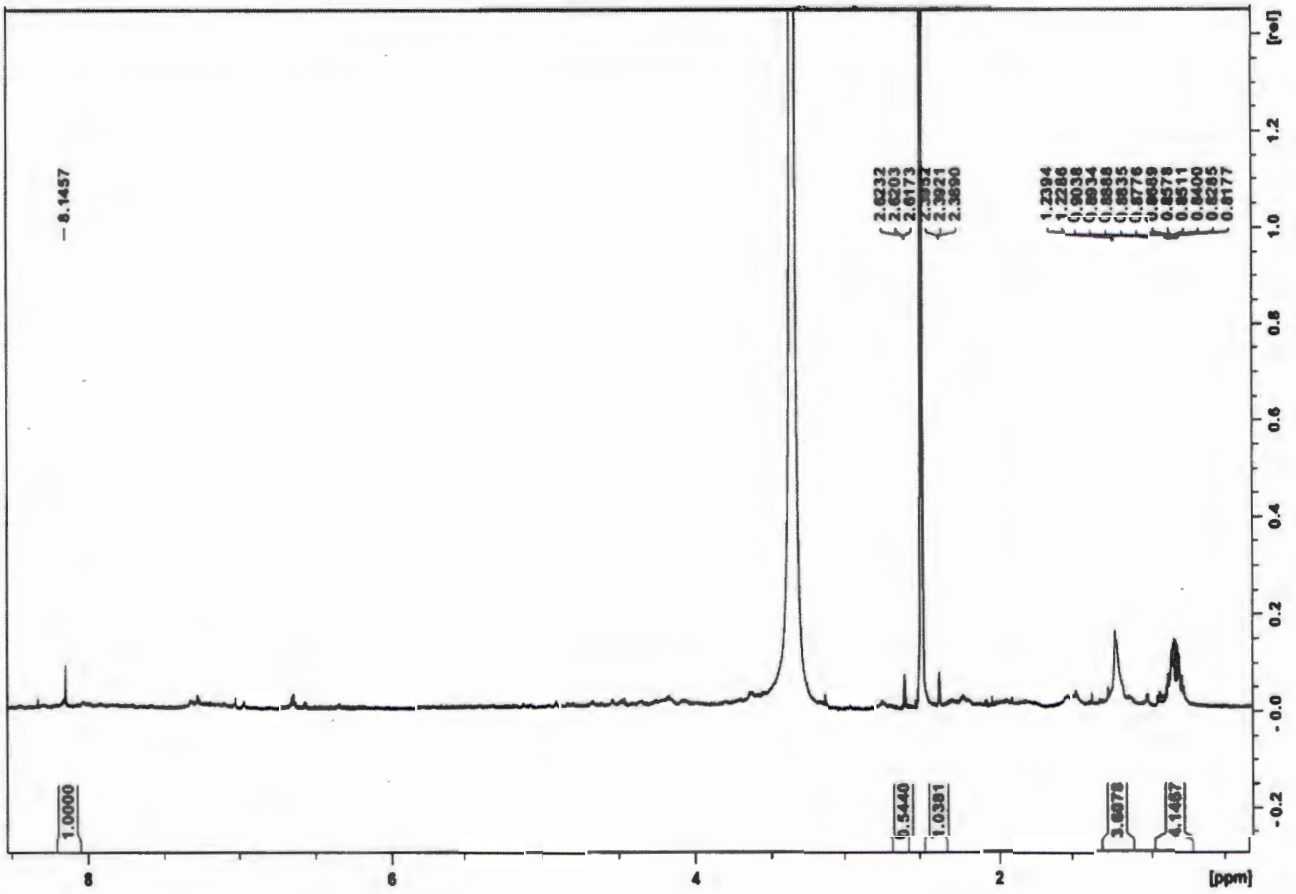
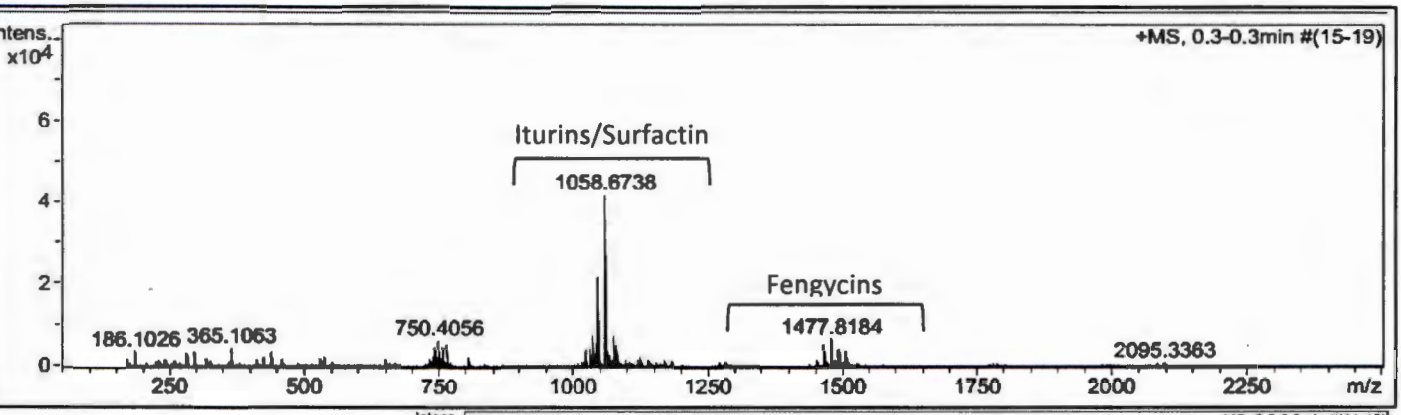


Fig. 7.6: NMR spectrum of BS10.5

7.3.4.3 ESI-MS analysis



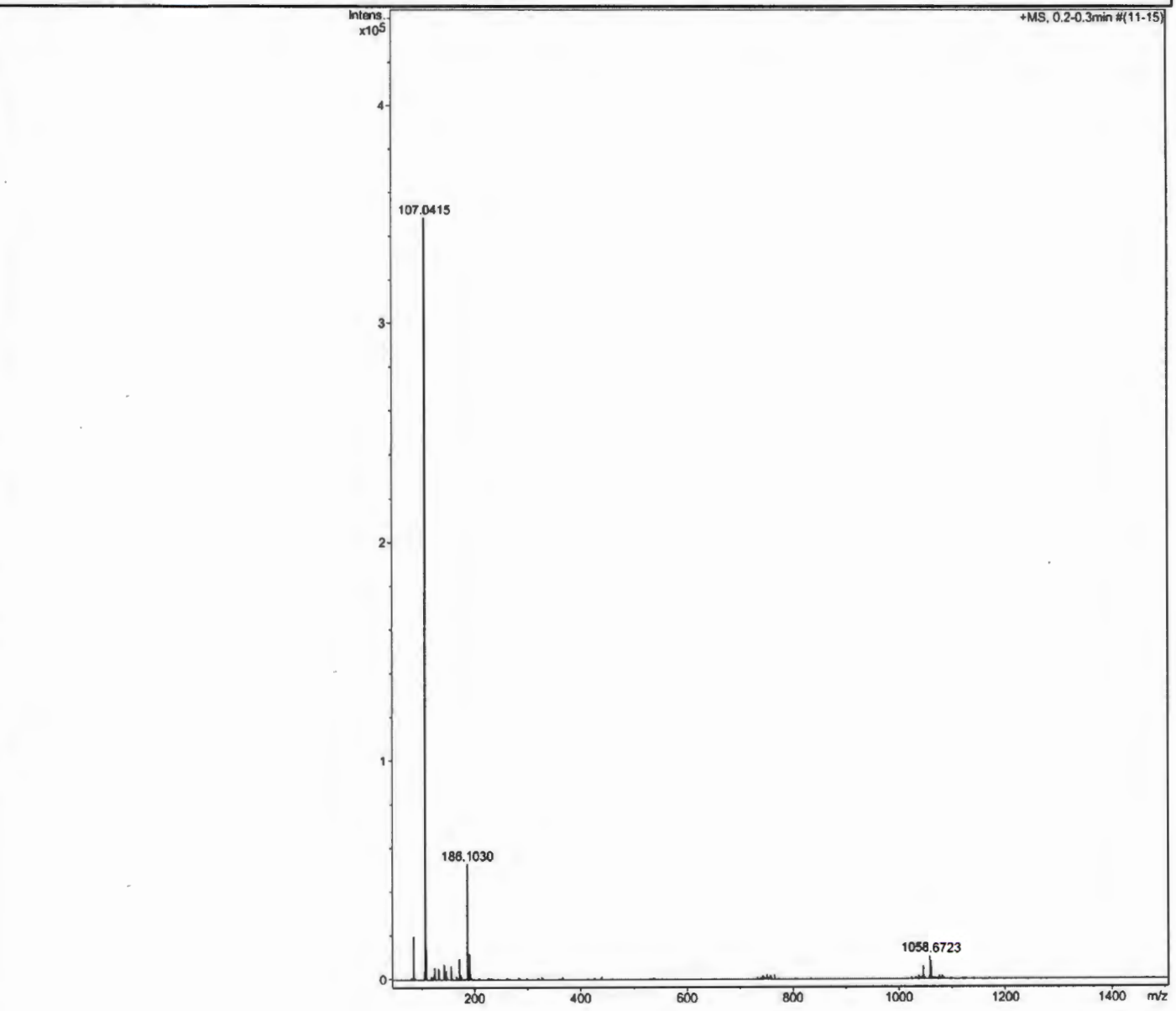


Fig. 7.7: Positive ESI-Q-TOF MS spectrum of lipopeptides extract of BS10.5 strain. Clusters of iturin, surfactin and bacillomycin (m/z 1058.6738, 1058.6740), fengycin (m/z 1477.8184), and an unidentified (m/z 2095.3363) molecular ion species are labeled.

The ESI-Q-TOF MS characterization showed that the BS10.5 lipopeptide extracts contained antifungal iturin, surfactin and fengycin families (Fig. 7.3a and 7.3c). Isolate BS10.5 had six mass ranges starting from m/z 186.1026, 365.1063, 750.4056, 1058.6738, 1477.8184 and 2095.3363. Mass 1058.6738 suggests the possible presence of iturin, surfactin and bacillomycin fragments, while the 1477.8184 represents the mass fragment of fengycin respectively (Ayed et al., 2014; Nam et al., 2015; Jasim et al., 2016). We arrived at this conclusion based on multiple comparison of literature and fragment matches. Fragments spectra of surfactin and its homologues (C13; 14 and 15) are mostly detected at 1,030.8, 1,044.8, 1,046.8, 1,058.8, 1,060.8, 1,074.8, that of bacillomycin D (C14, 15, 16 and 17) at 1,031.7, 1,045.7, 1,053.7, 1,059.7, 1,067.7, 1,069.7, 1,081.7, 1,083.7, 1,097.7, 1,095.7, 1,111.7, 1,527.8, 1,529.9, 1,543.8, that of fengycins (C15, 16, 17) at 1,449.9, 1,463.9, 1,471.9, 1,477.9, 1,485.9, 1,487.9, 1,491.8, 1,499.9, 1,501.9, 1,505.8, 1,513.9, 1,515.9 (Koumoutsi et al., 2004). The majority of these lipopeptides have a large variety of isoforms which sometimes present challenges in distinguishing them from each other (Chen et al., 2014). The ESI-MS was effective in detecting the presence of these compounds but the molecular mass at m/z 2095.3363 was insufficient for the identification of the likely compound.

7.3.5 Insights from exploration and *in silico* mining of *Bacillus velezensis* BS10.5 genome

The detailed *in silico* analyses revealed that the BS10.5 genome had 16 clusters which harbored over 76 homologous biosynthetic gene clusters (BGC). The genome has 4 non ribosomal peptide synthetase (NRPSs), two terpenes, 3 *trans*–AT PKS (transatpks), single lantipeptide, a

type 3 polyketide synthetase (T3pks), 2 *trans*–AT PKS-non ribosomal peptide synthetase, single bacteriocin-non ribosomal peptide synthetase cluster and two unidentified ketide sythethase (Table 7.3.2 and Figures 7.8a-7.8k). In figures 7.8a-7.8k, the colors symbolize different functional gene types: blue (transport-related genes); green (biosynthetic genes); red (regulatory genes) and grey (additional genes). In the anti-smash data, the highest number of biosynthetic genes were found in clusters 5, 6, 7, 14 (10 each), whereas, the PRISM result predicted 18 clusters in the BS10.5 genome. In table 7.4 we highlight the functions of the predicted biosynthetic compounds.

Table 7.3: Description and location of BGC in BS10.5 identified *in silico*

Cluster ID	Type	Position/Region	Numbers of BGC	Most Similar BGC predicted	Percentage similarity
Cluster 1 (218.374_ID_10360)	Terpene	42380 - 64263	1	unidefined	-
Cluster 2 (237.089_ID_10346)	Otherks	65373 - 106617	1	Butirosin	7% of genes similar
Cluster 3 (237.089_ID_10346)	Terpene	189395 – 210135	1	unidefined	-
Cluster 4 (237.089_ID_10346)	Transatpks	493558 – 579439	5	Macrolactin	100% of genes similar
Cluster 5 (237.089_ID_10346)	Transatpks - Nrps	808101 – 910775	10	Bacillaene	100% of genes similar

Cluster 6 (237.089_ID_10346)	Transatpks - Nrps	973335 - 1061819	10	Fengycin	86% of genes similar
Cluster 7 (228.907_ID_10356)	Transatpks	1 – 45825	10	Difficidin	53% of genes similar
Cluster 8 (228.907_ID_10356)	T3pks	161376 – 202527	1	unidefined	-
Cluster 9 (242.696_ID_1037)	Nrps	1 – 10331	1	unidefined	-
Cluster 10 (242.696_ID_1037)	Nrps	1 – 14180	2	Fengycin	13% of genes similar
Cluster 11 (232.565_ID_10370)	Transatpks	1 – 23720	7	Difficidin	26% of genes similar
Cluster 12 (246.163_ID_10350)	Nrps	516770 – 569433	1	unidefined	-
Cluster13 (263.57_ID_10354)	Nrps	1 – 25748	3	Surfactin	47% of genes similar

Cluster 14 (265.136_ID_10348)	Bacteriocin - Nrps	1981 - 68772	10	Bacillibactin	100% of genes similar
Cluster 15 (265.136_ID_10348)	Other	574581 – 615999	6	Bacilysin	100% of genes similar
Cluster 16 (265.136_ID_10348)	Lantipeptide	766502 – 789690	1	Mersacidin	90% of genes similar

7.3.5.1 Molecular annotation of the predicted sixteen clusters identified in the *Bacillus velezensis* BS10.5 genome.

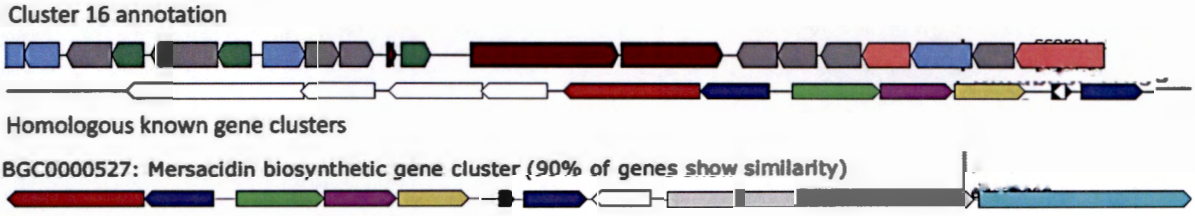


Fig. 7.8a: Mersacidin was the major BGC predicted from cluster 16 of the BS10.5 genome

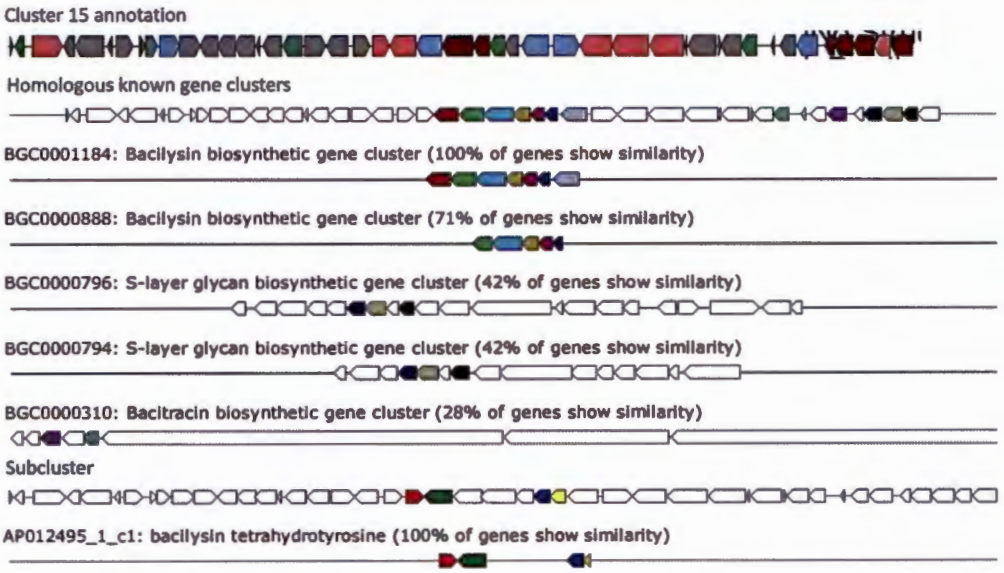


Fig. 7.8b: Bacilysin, S-layer glycan, Bacitracin were the major BGC predicted from cluster 15 of the BS10.5 genome

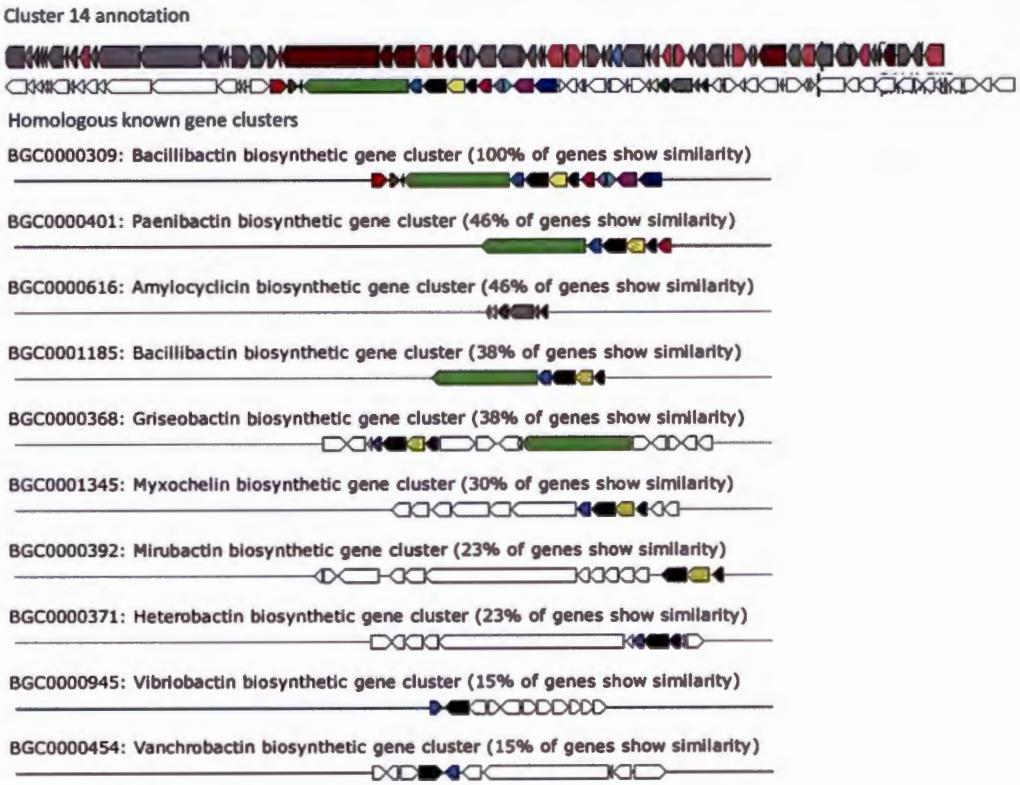


Fig. 7.8c: Major BGC predicted from the cluster 14 of the BS10.5 genome

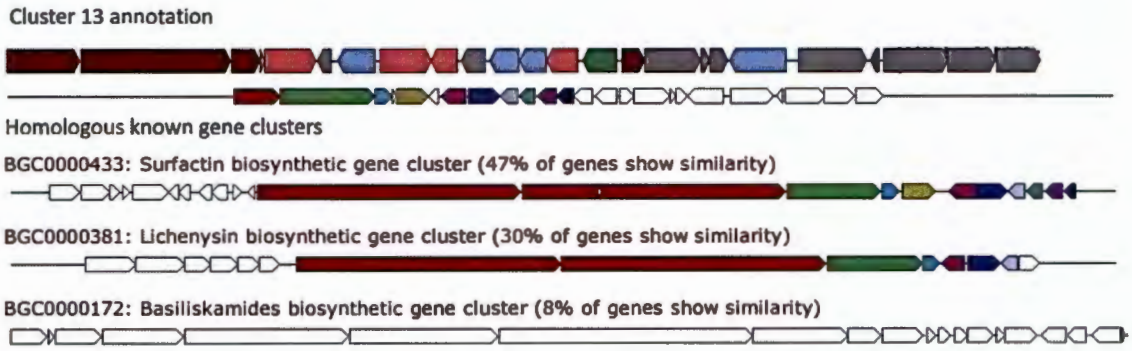


Fig. 7.8d: BGC predicted from cluster 13 of the BS10.5 genome includes surfactin (sfp gene) a major lipopeptide detected during amplification of PCR product of BS10.5 DNA extract.

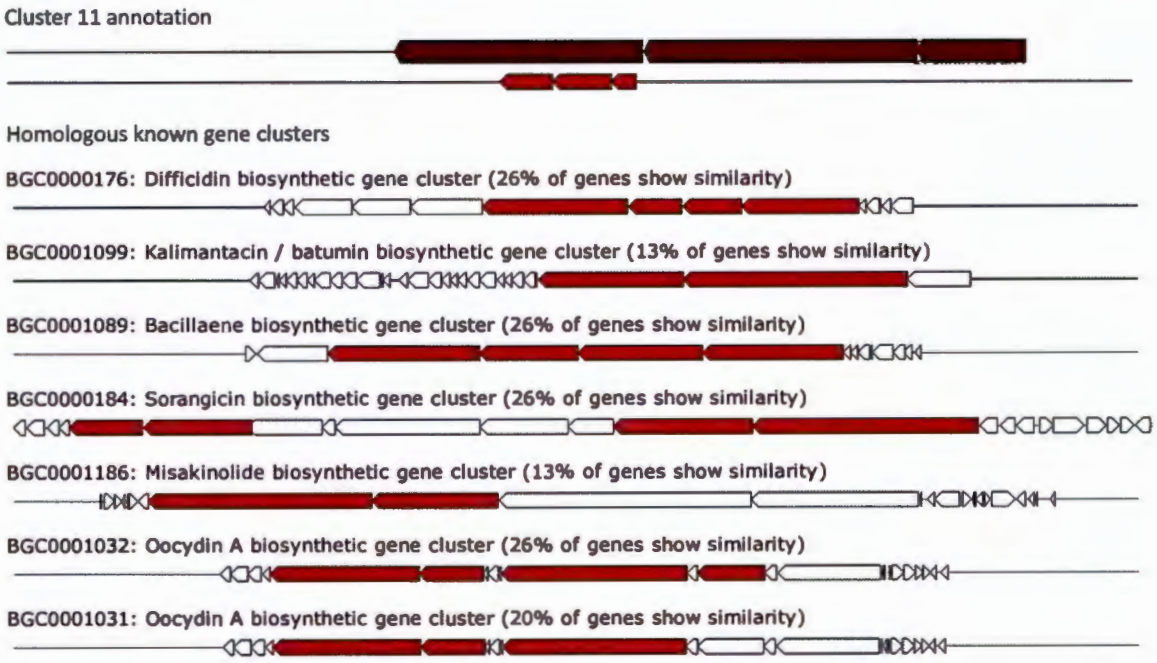


Fig. 7.8e: Major BGC predicted from the cluster 11 of the BS10.5 genome. The BGC showed similarity percentages lesser than 30.

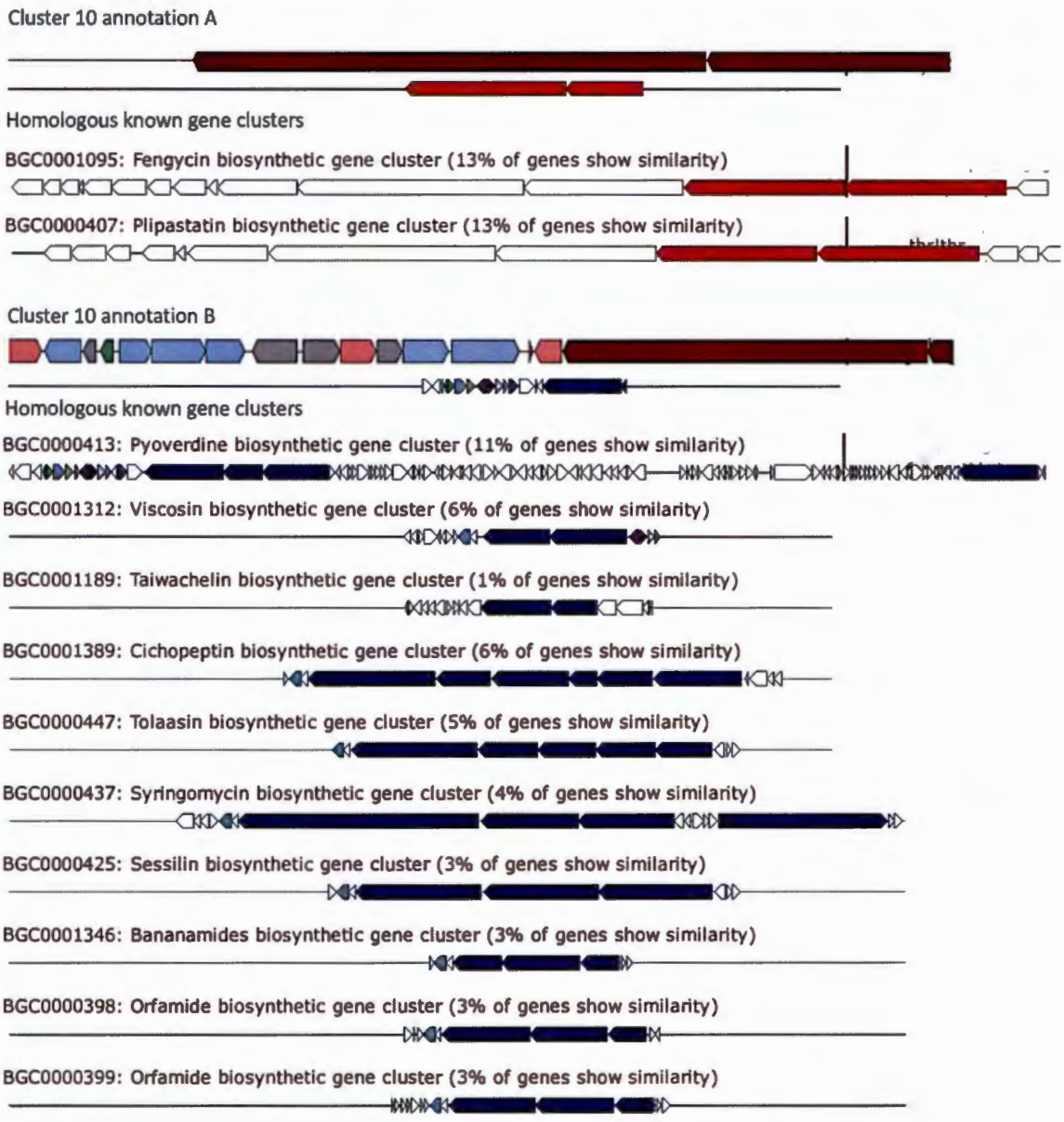


Fig. 7.8f: Major BGC predicted from cluster 10 of the BS10.5 genome which had 2 different sub annotations. Fengycin was predicted at 13% similarity which was earlier detected during PCR-Gel electrophoresis.

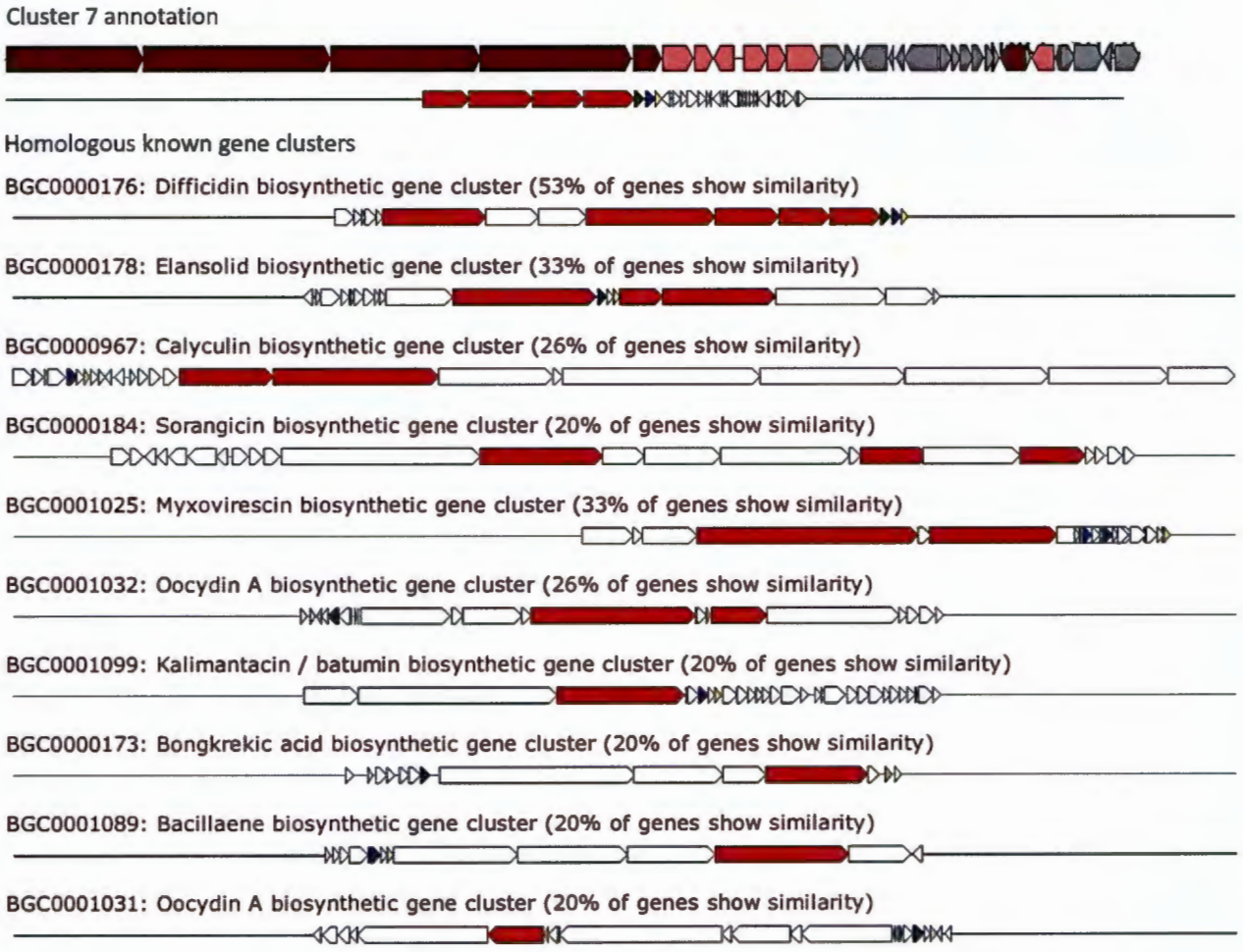


Fig. 7.8g: Major BGC predicted from cluster 7 of the BS10.5 genome

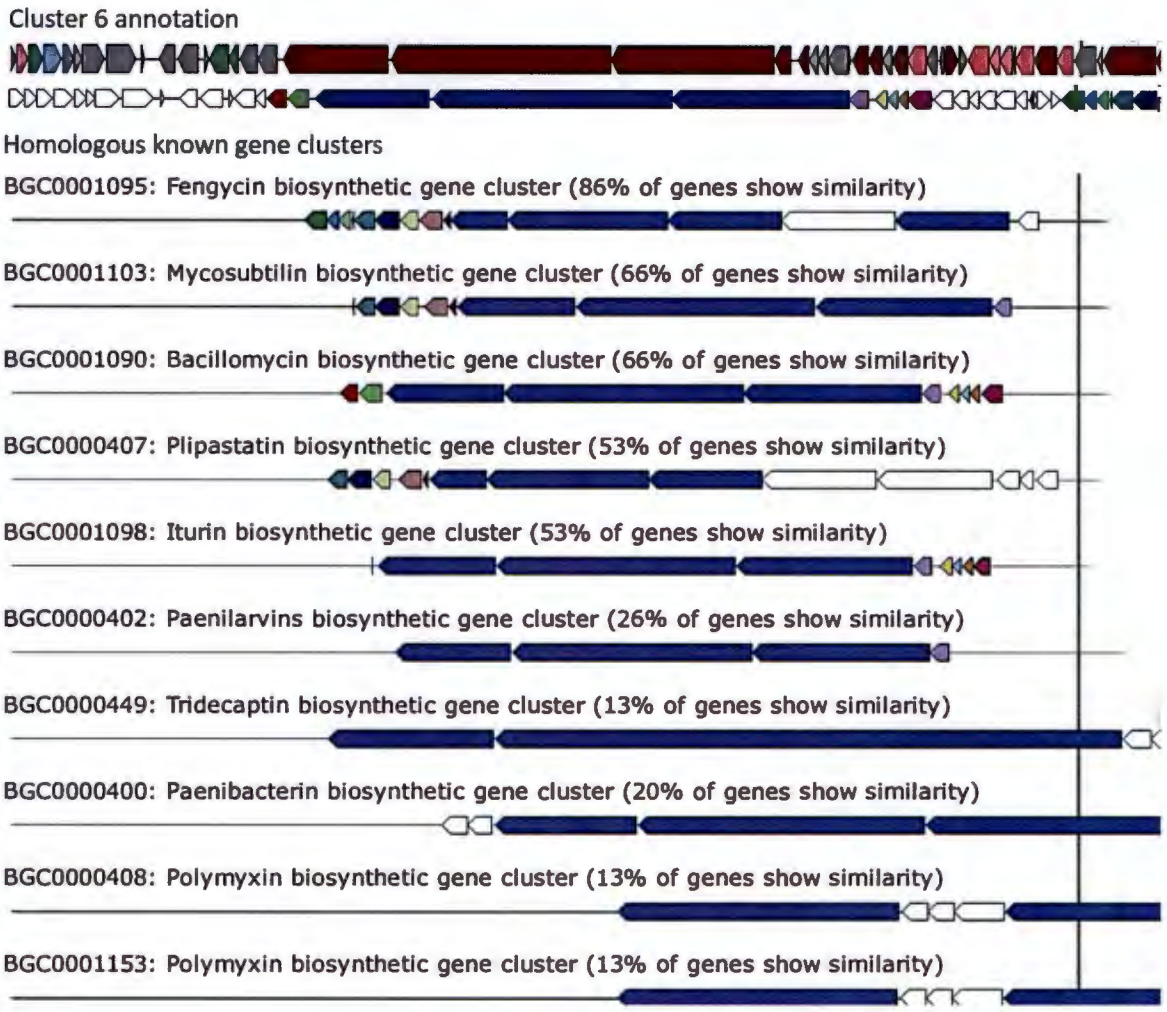


Fig. 7.8h: Major BGC predicted from cluster 6 of the BS10.5 genome.

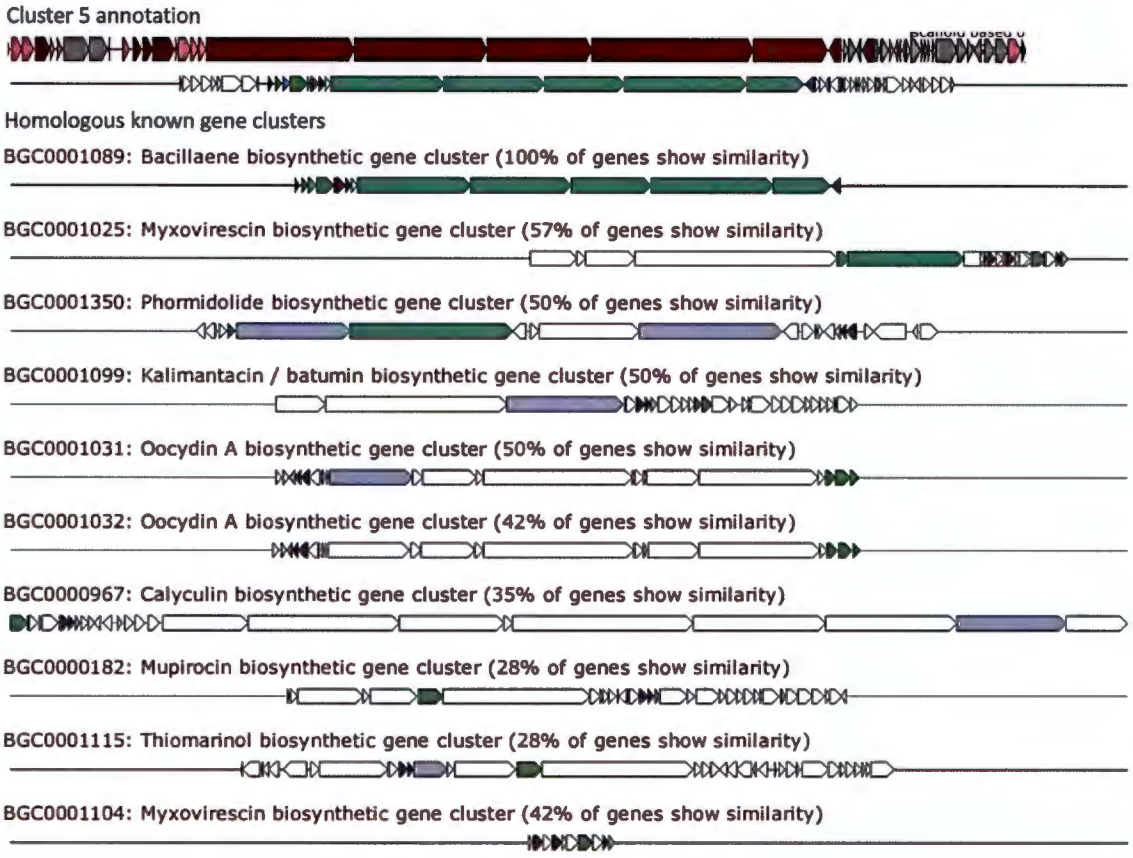


Fig. 7.8i: Major BGC predicted from cluster 5 of the BS10.5 genome

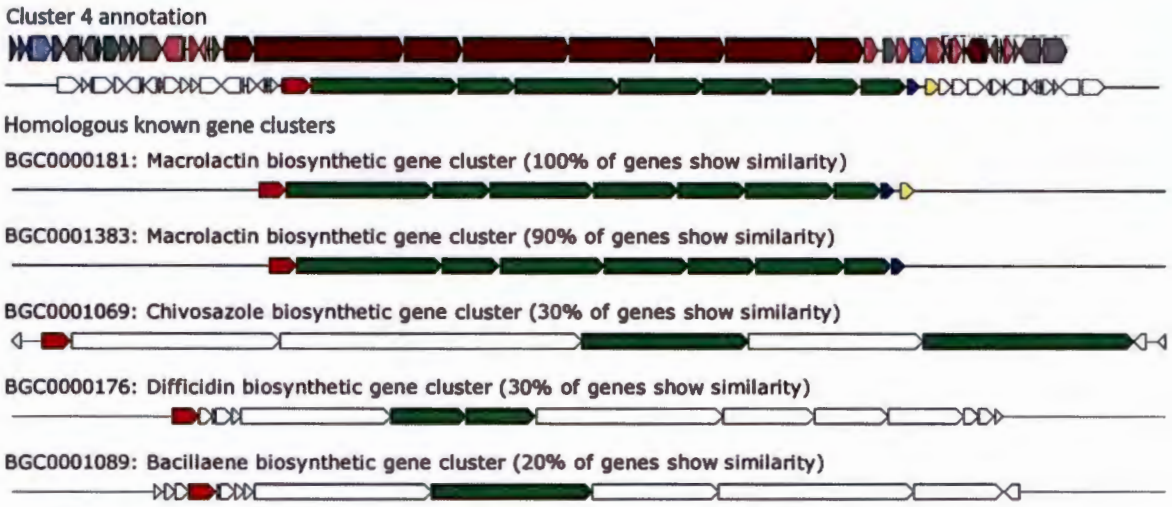


Fig. 7.8j: Major BGC predicted from cluster 4 of the BS10.5 genome

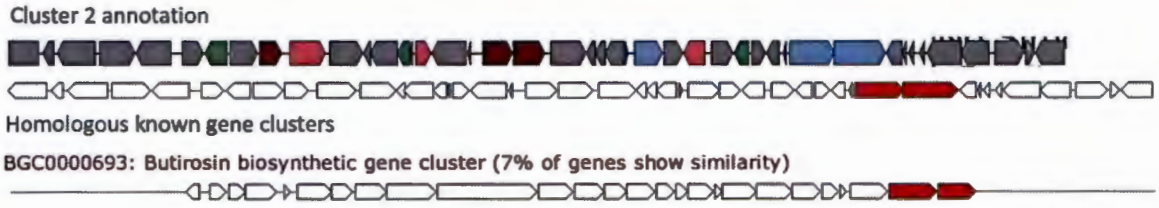


Fig. 7.8k: Major BGC predicted from cluster 2 of the BS10.5 genome

Table 7.4: Overview of the functions of the BCs predicted in the BS10.5 genome

Biosynthetic compound	Cluster located	Biomedical/Biocontrol function	Reference
Mycosubtilin	6	Antifungal, hemolytic and limited antibacterial activity	Leclère et al., 2005; Stein (2005); Duitman (2007)
Fengycin and Plipastatin	6	Broad-spectrum antifungal and antitumoral agent	Vanittanakom et al., (1986); Cochrane et al., (2016)
Surfactin	13	Antibacterial, antifungal, antiviral, antimycoplasma, antitumoral, insecticidal, anticoagulant activities and enzyme inhibitors	Ongena et al., (2007); Mnif et al., (2015)
Iturin	6	Antibacterial and antifungal activity	Ongena et al., (2007); Dunlap et al., (2013)
Polymyxin	6	Antibacterial, antifungal and Immuno-modulating activity	Cochrane et al., (2016)
Sessilin	10	Antifungal activity	D'Aes et al., 2011; Olorunleke et al., 2015
Bananamides	10	Unspecified	Nguyen et al., (2016)
Cichopeptin	10	Limited information	Huang et al., (2015)
Viscosin	10	Biosurfactant	Alsohim et al., (2014); Bonnichsen et al., (2015)

Taiwachelin, Tolaasinand Orfamide	10	Iron chelation, therapeutic peptide, insecticidal biosurfactant and elicitor of induced systemic resistance	Rainey et al., (1991); Andolfi et al., 2008; Kreutzer et al., (2012); Jang et al., (2013); Ma et al., 2017
Mersacidin	16	Antibacterial	Abriouel et al., (2010)
Bacitracin and Bacilysin	15	Limited use as animal growth promoter, topical antibiotic antibacterial and antifungal	Phillips (1999); Mousa et al., (2015)
Bacillibactin, Paenibactin, Griseobactin, Heterobactin, Mirubactin, Myxochelin, Vanchrobactin	14	Iron chelation and anticancer agent	Silakowski et al., (2000); Balado et al., (2008); Patzer and Braun (2010); Sandy et al., (2010); Wen et al., (2011); Giessen et al., (2012); Segond et al., (2014)
Amylocyclicin	1	Antibacterial and Plant growth promoter	Scholz et al., (2014)
Lichenysin	13	Biosurfactant	Grangemard et al., (1999)
Basiliskamides	13	Antifungal	Theodore et al., (2014)
Difficidin	11, 7, 4	Broad spectrum antibacterial compound	Chen et al., (2009); Mousa et al., (2015)
Kalimantacin/batumin and Oocydin A	11, 7, 5	Antibacterial and antifungal haterumalide	Tokunaga et al., (1996); Mattheus et

			al., 2010; Matilla et al., (2015)
Sorangicin	11, 7	Antibacterial macrolide antibiotic	Campbell et al., (2005)
Elansolid	7	Antibiotic (Bactericidal)	Steinmetz et al., (2010)
Myxovirescin	7, 5	Antibiotic (Bactericidal)	Xiao et al., (2012);
Phormidolide, Thiomarinol and Mupirocin	5	Antitumor agent and antibacterial metabolite/clinical antibiotic	Lorente et al., (2014); Murphy et al., (2014); Mousa et al., (2015)
Paenilarvins, Tridecaptin and Paenibacterin	6	Antifungal, antitumor agent and Antibiotic (Bactericidal)	Huang and Yousef 2014); Hertlein et al., (2016); Cochrane et al., (2016)

7.3.5.2 Pan-genome comparison, WGS nucleotide blast and NCBI biosynthetic gene

Firstly, we carried out a genome blast search of BS10.5 to determine its genetic relatedness, then we blast searched the genome against biosynthetic gene clusters for lipopeptide and constructed the phylogenetic trees (Figure 7.9a and 7.9b). Thirdly, the pan-genome comparisons of four established PGP *Bacillus* spp. in relation to BS10.5 strains confirmed the distinctive status of BS10.5 and its genetic relatedness (Figure 7.9c).

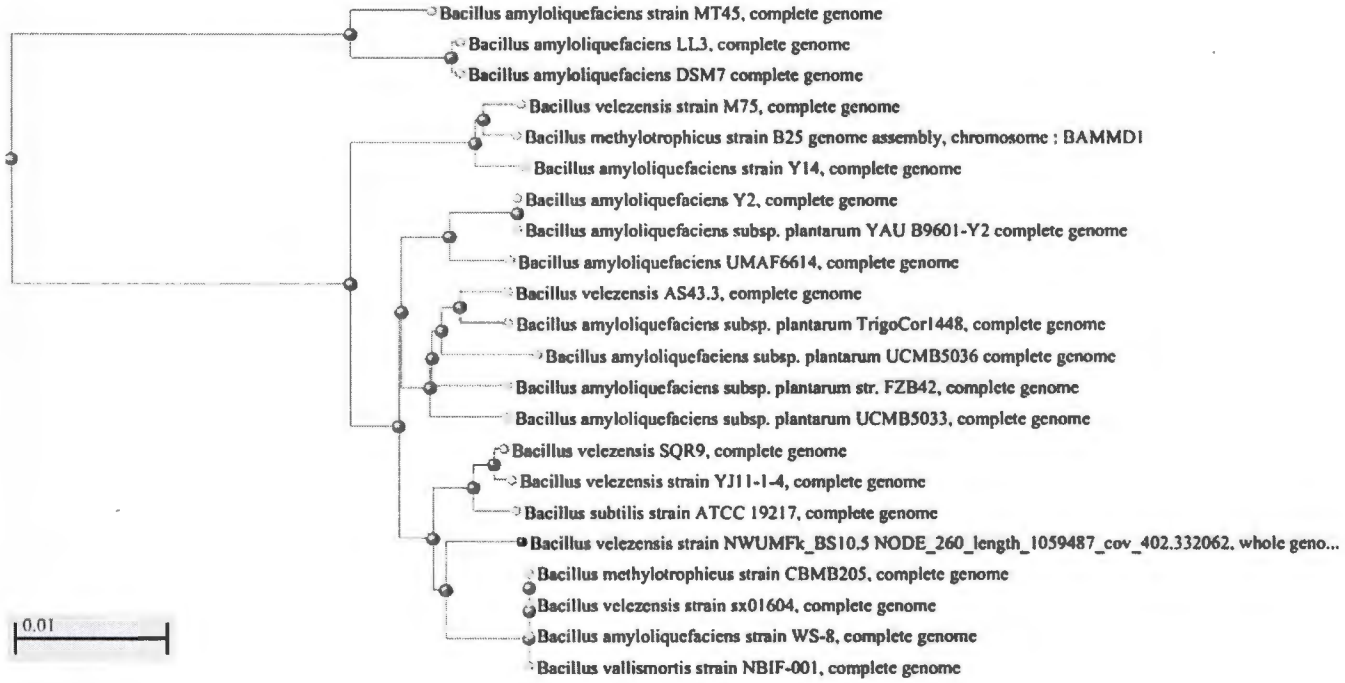


Fig. 7.9a: Phylogenetic tree from pangenomic sequence of closely related *B. veleznesis* strains

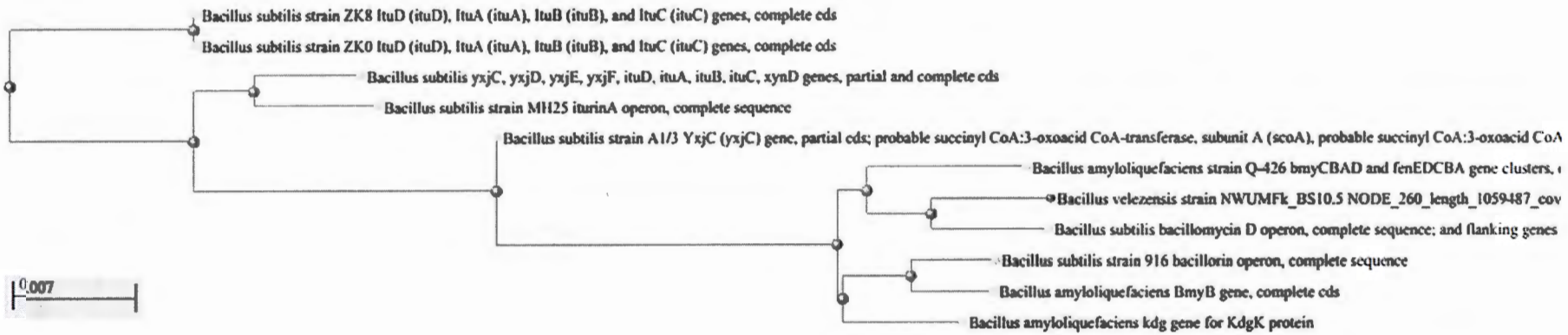


Fig. 7.9b: Pangenomic tree of *B. veleznesis* strains. A node portion of the Genome of BS10.5 clustered with other *Bacillus* strains that harbored gene required for the synthesis of known lipopeptide genes. The node_260_1059487 clustered directly with *B. subtilis* bacillomycin D operon and *B. amyloliquefaciens* strain Q-426 fengycin gene clusters.

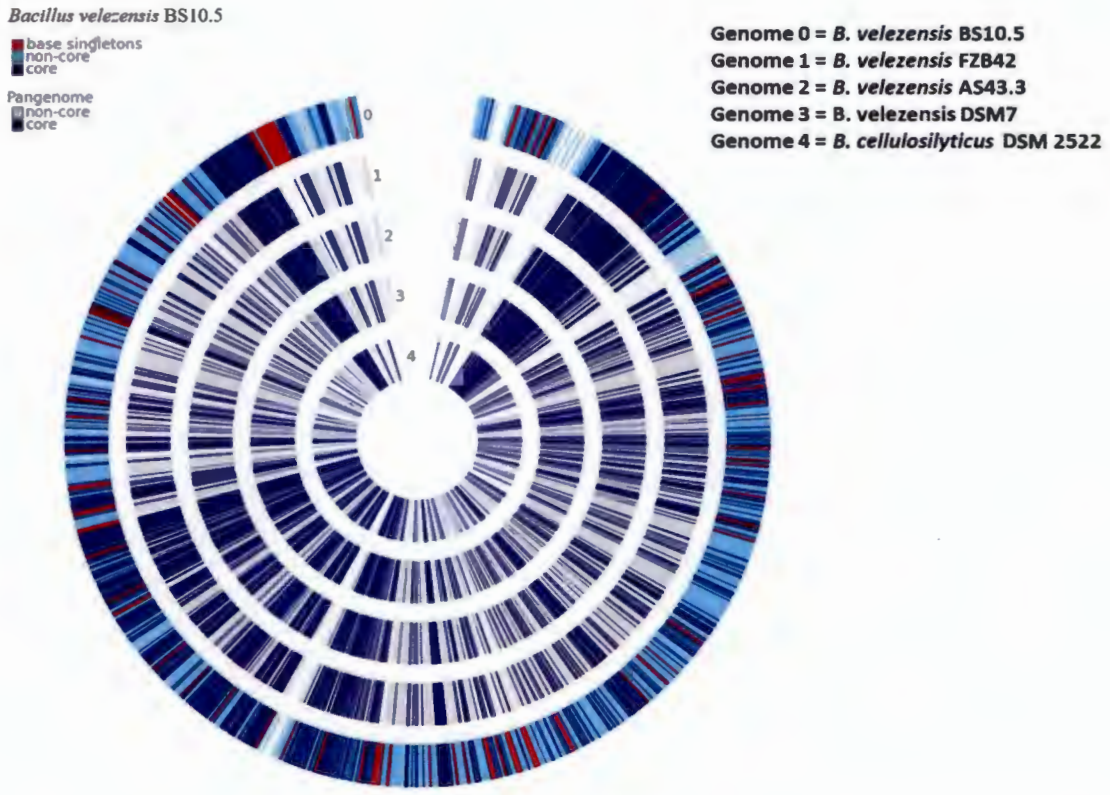


Fig. 7.9c: Pangenomic atlas of BS10.5 with closely related *B. veleznesis* strains and an out group *B. cellulosilyticus* DSM 2522 (genome 4).

7.3.5.3 Metabolic modelling of the BS10.5 genome

Using the KEGG platform, we constructed a genome-scale metabolic model for the BS10.5 genome based on its annotations. The final model includes 1558 reactions, 1559 compounds, and 1000 genes. Forty-three new reactions were added to the model, while 3 existing reactions were made reversible (Data in Appendix). We highlight a few of the pathways responsible for the biosynthesis of beneficial compounds Table 7.5 and Figures 7.10a - 7.10. The result shows the genomic robustness of the BS10.5 and its capacity to synthesize numerous compounds and biosynthetic intermediates through diverse metabolic pathways. Twenty-eight significant compounds were predicted from the major metabolic pathways constructed.

Table 7.5: Biosynthetic pathways and compounds identified from the metabolic model

Biosynthetic pathway	Compound synthesized	Beneficial significance
Tetracycline biosynthesis	Oxytetracycline and tetracycline	Biomedical antibiotic
Brassinosteriod biosynthesis	Brassinolide	Phytohormone
Puromycin biosynthesis	O-beta-D puromycin	Antibiotic
Zeatin biosynthesis	Xylosylzeatin, zeathin and lupinate	Phytohormones
Anthocyanin biosynthesis	Cyanidin, malvidin, delphinidin, malonylshisonin and pelargonidin	Plant pigments
Benzoxazinoid biosynthesis	DIMBOA	Plant antibiotic
Vancomycin biosynthesis	Chloroeremomycin and vancomycin	Antibiotics
Ansamycins biosynthesis	Rifamycin and protorifamycin	Antibiotics
Chondroitin biosynthesis	Chondroitin	Medical supplement

Monoterpenoid biosynthesis	Asperuloside and myrcene	Plant defense compounds and anti-*Helicobacter pylori*
Betalain biosynthesis	Gomphrenin-1, lampranthin-2, celosianin-2, 2-decarboxybetanidin, betalamic acid and miraxanthin V	Novel antioxidants and food additives
Novobiocin biosynthesis	Albamycin and maltol	Antibiotic, antiioxidant and flavour enhancer

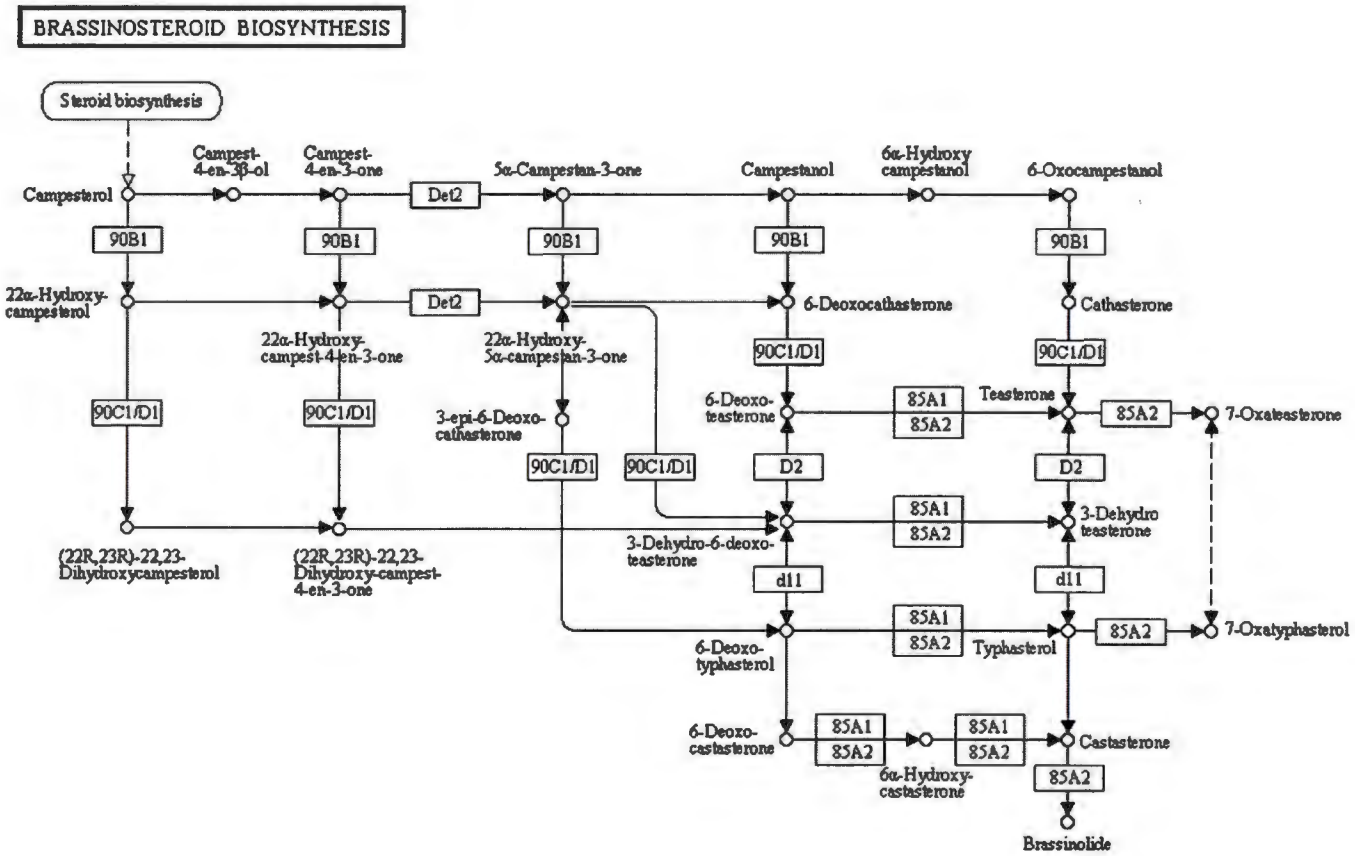


Fig. 7.10a: Metabolic pathway for the synthesis of Brassinosteroid.

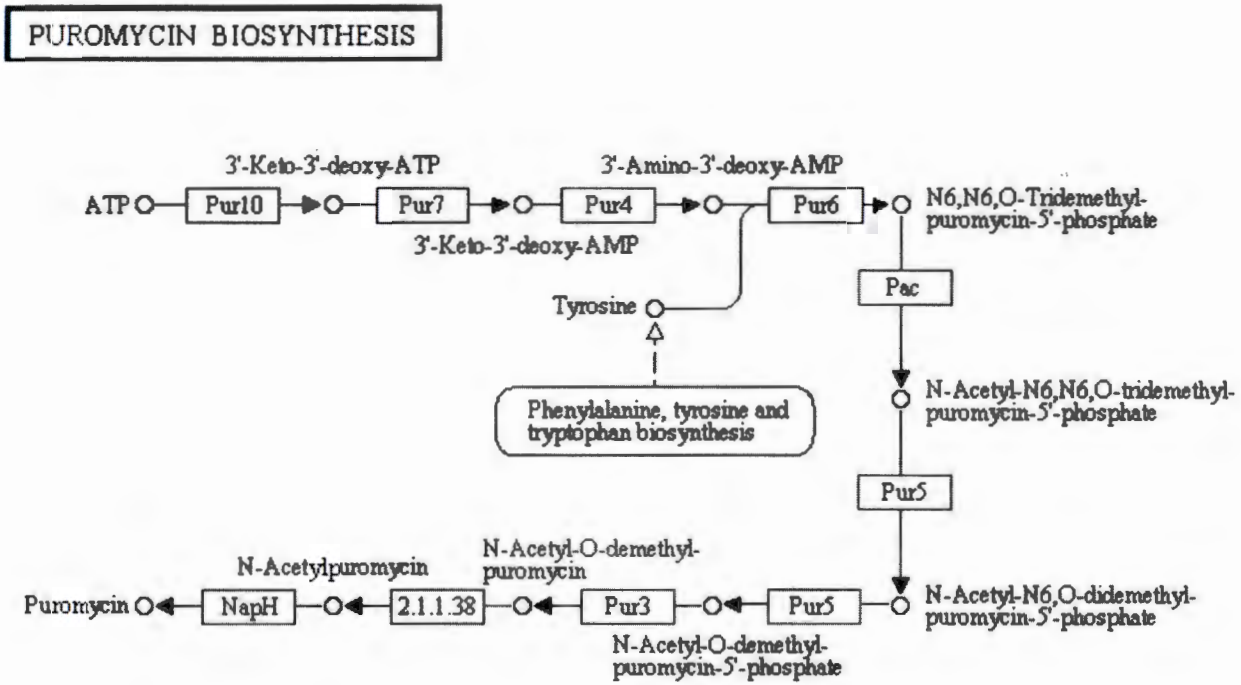


Fig. 7.10b: Metabolic pathway for the biosynthesis Puromycin.

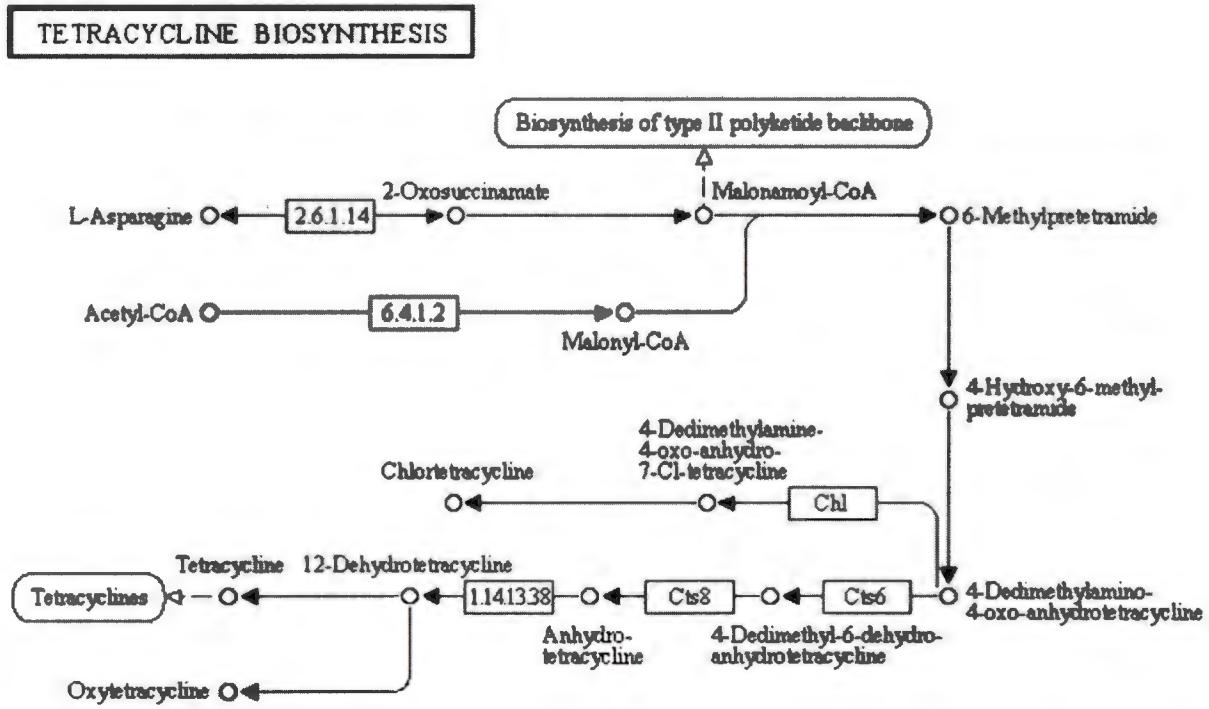


Fig. 7.10c: Metabolic pathway for the biosynthesis of Tetracycline.

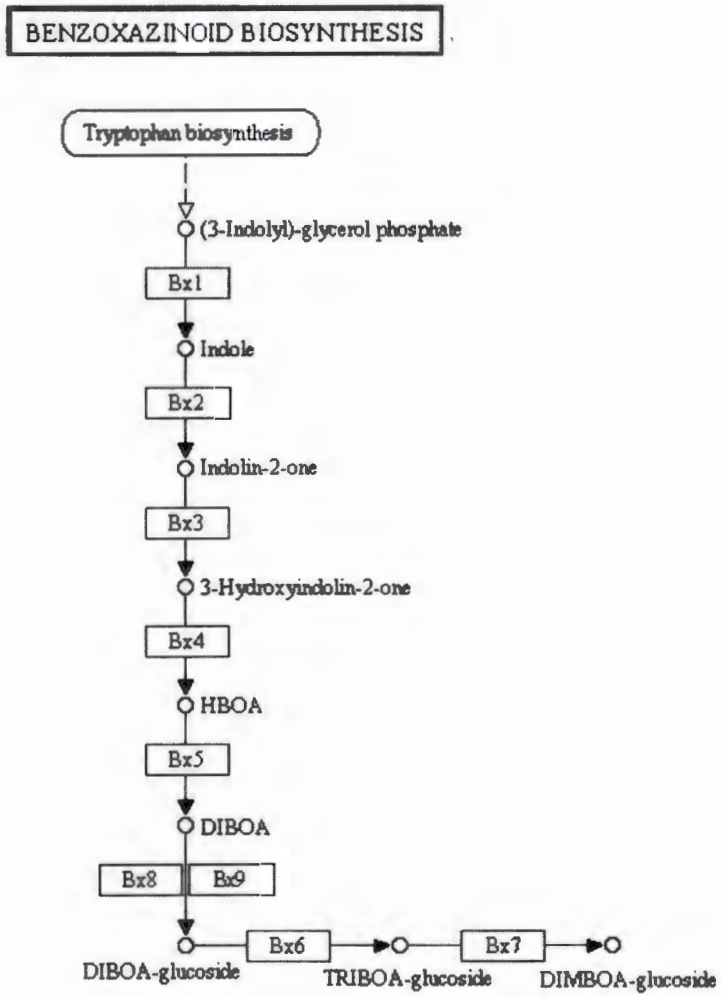


Fig. 7.10d: Metabolic pathway for the Benzoxazinoid biosynthesis.

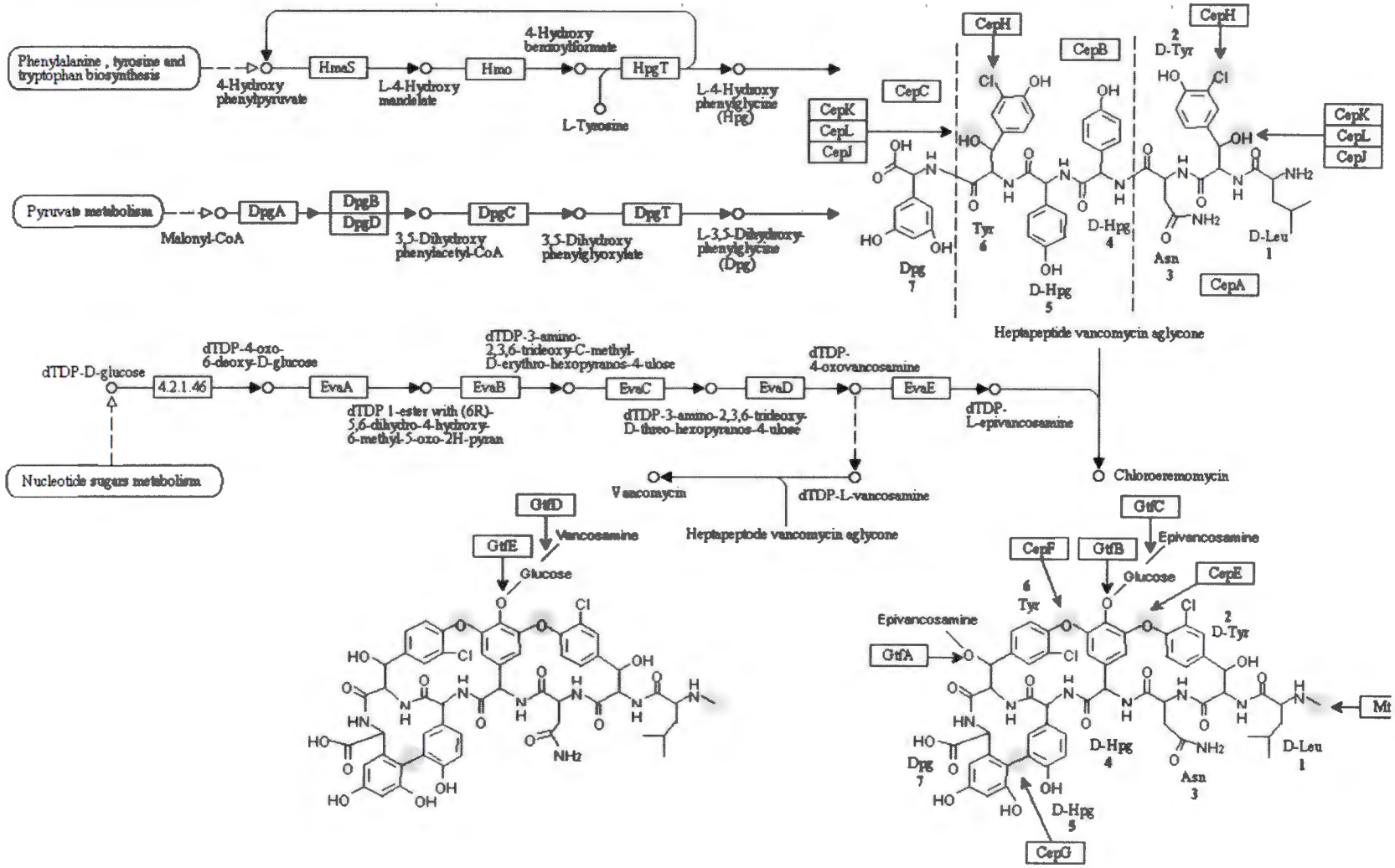


Fig. 7.10e: Metabolic pathway for the biosynthesis of vancomycin antibiotics.

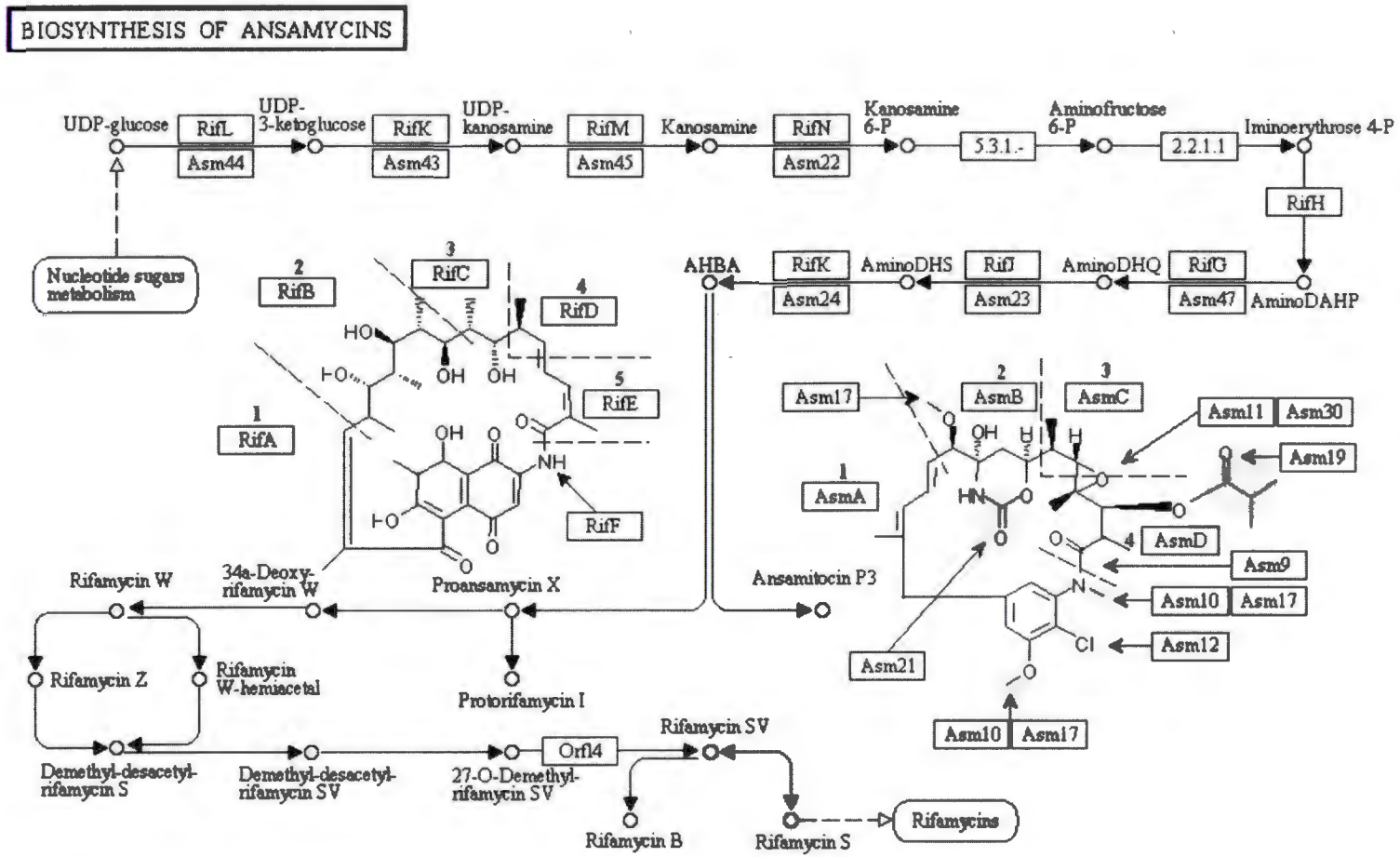


Fig. 7.10f: Metabolic pathway for the biosynthesis of anamycins

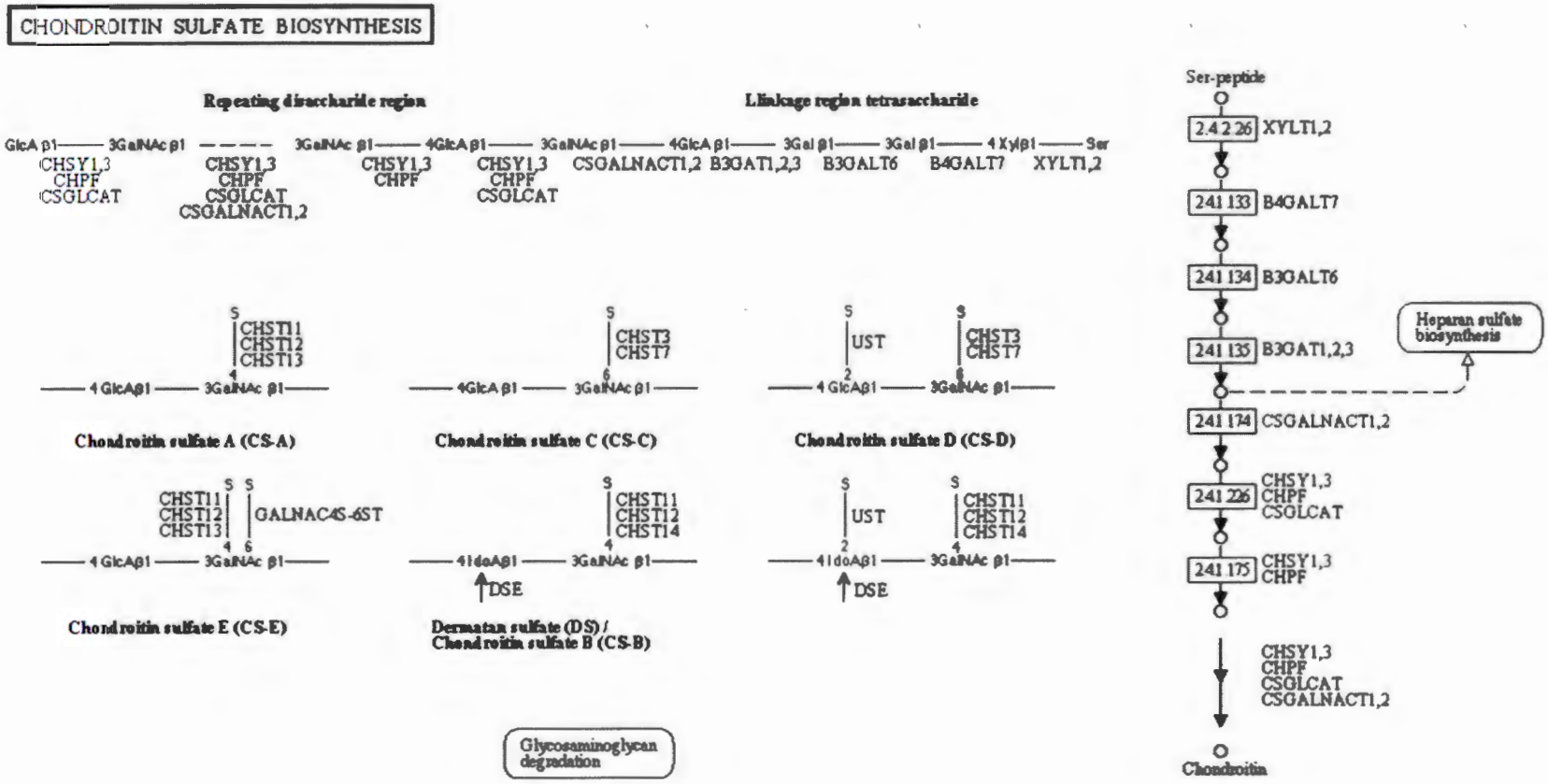


Fig. 7.10g: Chrondoitin biosynthesis metabolic pathway.

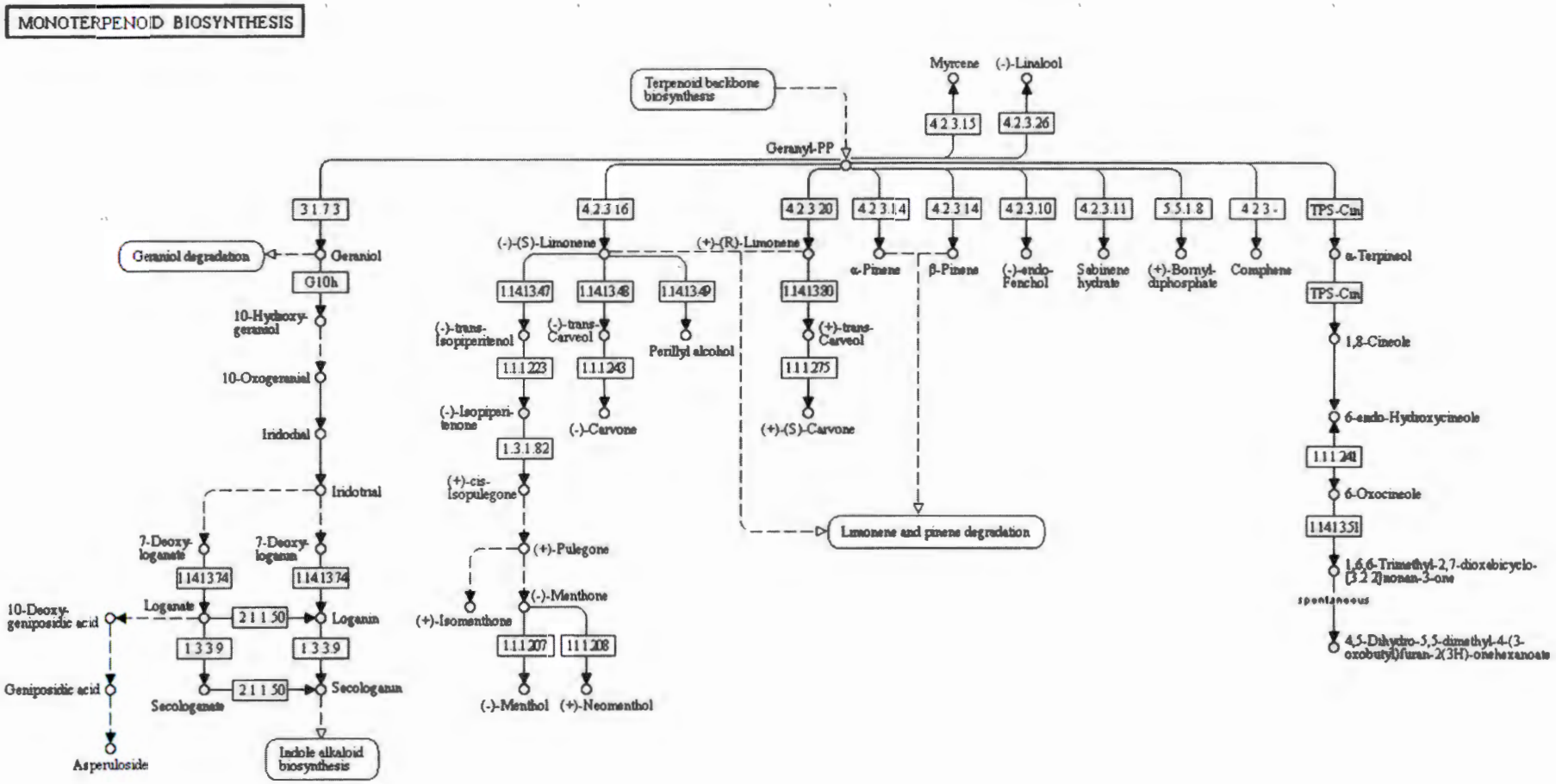


Fig. 7.10h: Biosynthetic pathway for the production of the monoterpenoids

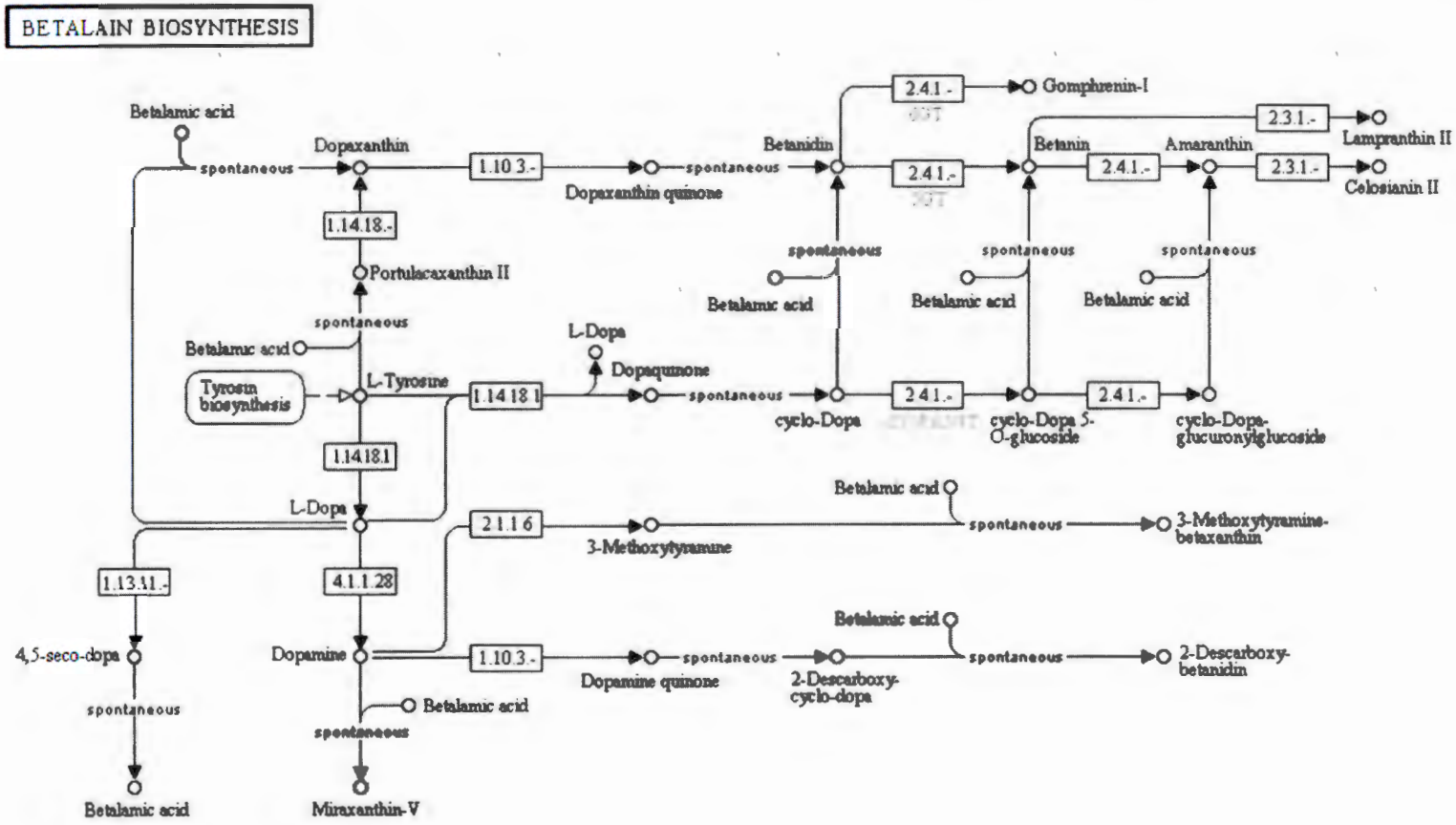


Fig. 7.10i: Betalain biosynthetic pathway

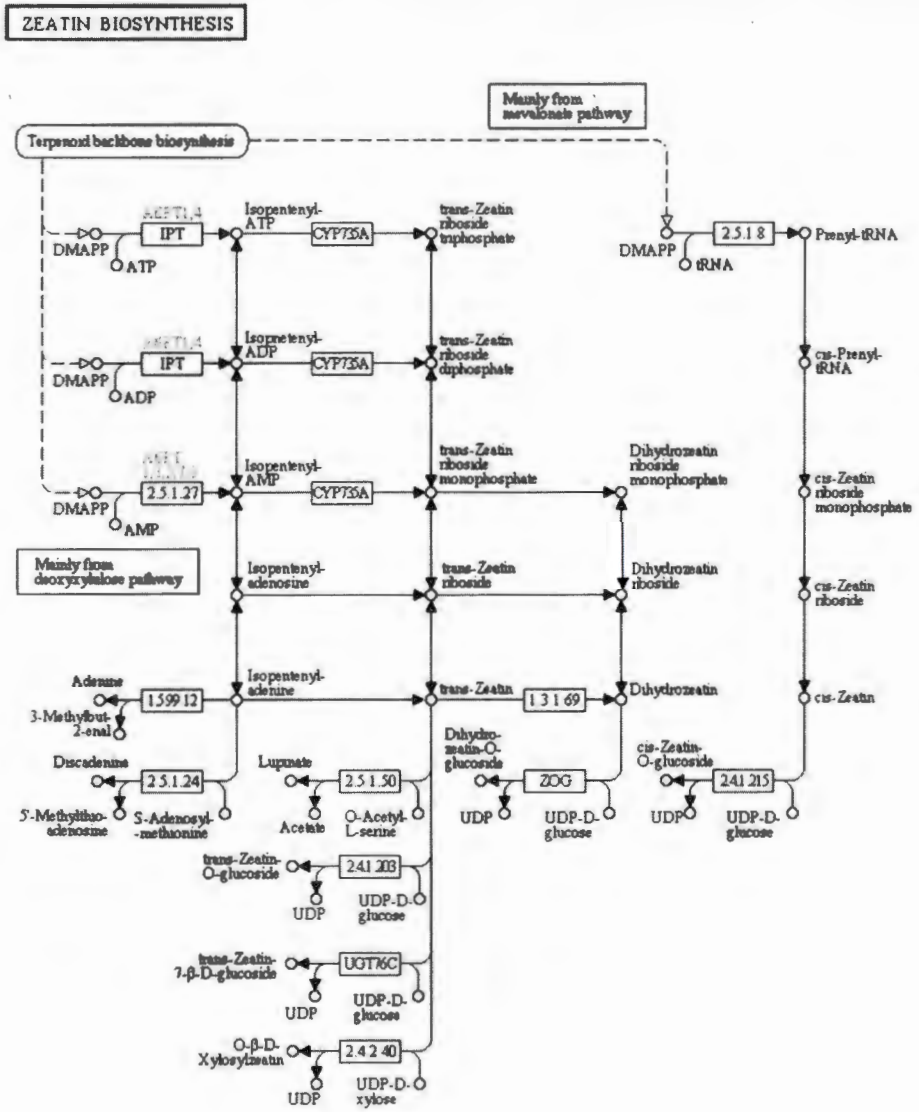


Fig. 7.10j: Zeatin biosynthesis pathway

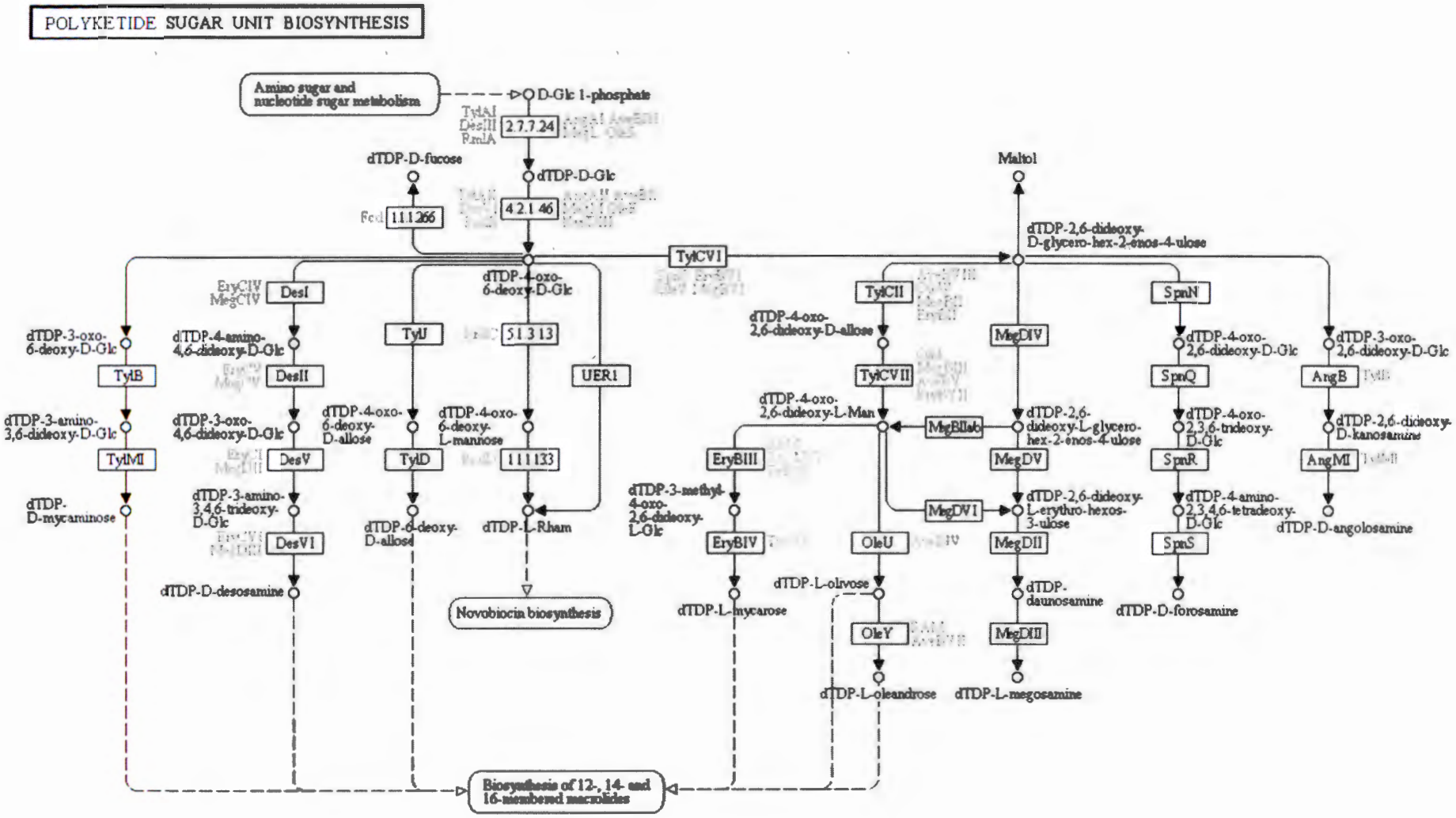


Fig. 7.10k: Metabolic pathway for the biosynthesis of the polyketide sugars

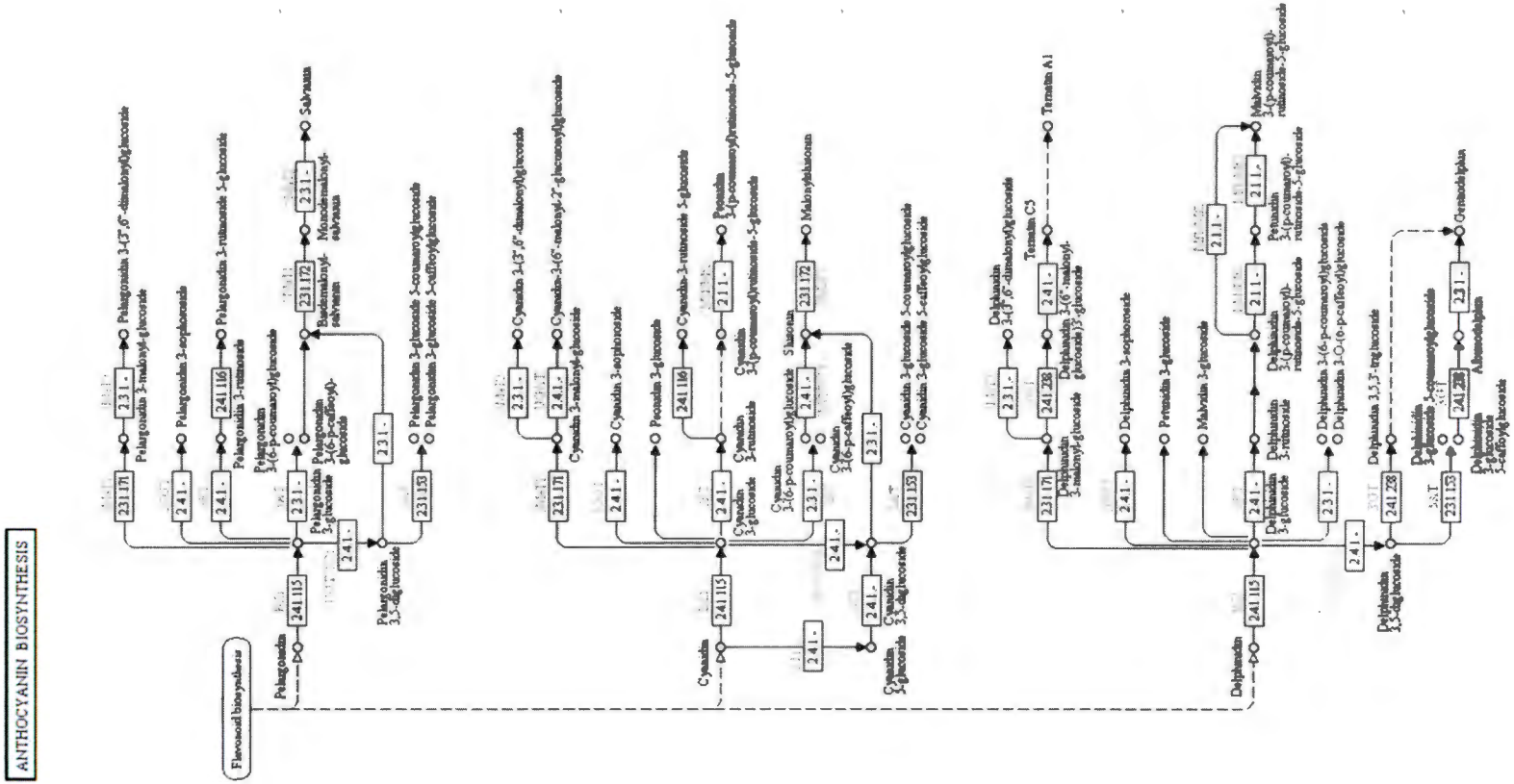


Fig. 7.10i: Metabolic pathway for anthocyanin biosynthesis

Discussion

Culture free supernatants extracted from bacterial antagonists have been used to express the antimicrobial activity through several modes of application such as seed treatments and pour plate mixtures (Abd El et al., 2015; Abdallah et al., 2015; Ali et al., 2016). The culture free supernatant of potential microbial inoculant can be of beneficial importance if it is produced in sufficient volume during fermentation processes. Culture free supernatants always do contain diverse metabolites such as indole-acetic acid (IAA), antibiotics, lytic enzymes, iron chelating compounds and hormones depending on the microbial growth phase and culture conditions (Ahmad et al., 2008; Chen et al., 2014). Secretion and accumulation of surfactin occurs at the logarithmic phase while the synthesis of iturin occurs during the stationary phase of growth (Leclere et al., 2005). Furthermore, bacterial cell-free supernatants, crude extracts, cell cultures, volatiles and purified extracts all demonstrate variable levels of suppressive activity when they are used during bioassays (Baysal et al., 2013).

The influence of solvents used in diluting or dissolving them during antimicrobial assays has been a topic of discussion. We employed PBS as our diluting solvent here because it had no influence on the activity of the extract. It is also important to determine the minimum inhibitory concentration of an antimicrobial agent. Here we employed a dose dependent assay to determine the concentrations at which the pathogens were most sensitive. Growth of the pathogens retarded as clearing zones were seen increasing relative to increased concentration of the extract. However, at 30 µl three of the pathogens (PA, MC and KP) showed no sensitivity. This gives an indication of the lowest possible concentration at which the pathogens will remain susceptible. Dose dependent effects of lipopeptides have been reported elsewhere (Falardeau et al., 2013). A detailed

assay might however be needed to confirm the minimum inhibitory or cidal dose of the extract. Commercial fungicides containing nystatins, triazoles and amphotericins have been used as controls to compare sensitivity of *Fusarium* to fungal antibiotic (Arutchelvi et al., 2010; Girija et al., 2014) and *Fusarium* spp. are less susceptible to amphotericin than nystatins and triazoles (Deepak and Jayapradha, 2015). Our lipopeptide extract showed excellent inhibitory activity in comparison to the fungicides we utilized in this study. Our results correlate with other reports showing comparative multi-pathogenic effects of crude, partially purified or purified extracts of bacterial antagonist (Chen et al., 2010; Ye et al., 2012; Mousa et al., 2015; Altaee et al., 2016).

The mechanism of action of these microbial lipopeptides is advantageous compared to conventional antibiotics in that they disrupts the cell membranes of phytopathogens while conventional antibiotics tend to penetrate the cell for activity which is prone to resistance due to pathogen efflux systems. Action mechanisms of microbial lipopeptides reduce the tendency of microbes developing resistance to them (Song et al., 2013). We had earlier amplified the genes responsible for the synthesis of surfactin, bacillomycin, iturin D, fengycin D in *Bacillus* BS10.5 amplicons during PCR analysis. The FTIR, NMR and ESI-MS results further provide evidence that the three most reported antimicrobial lipopeptides in *Bacillus* spp. (surfactin iturin, fengycin) are responsible for the biocontrol activity of BS10.5. Reports showing non synergistic and synergistic activity of lipopeptides against fusarium members are available (Koumoutsi et al., 2005; Xu et al., 2013; Chen et al., 2016) and *Bacillus* spp. with conserved genes for producing multiple lipopeptides are not rare (Jemil et al., 2017).

The result of our analysis of the BS10.5 genome shows similarity identity above 85% for some biosynthetic genes clusters (BGC). Technically, BGC or biosynthetic genes are considered

to be present when similarity index is 65% and above (Van Der Voort et al., 2015). However, our report here also shows that a low percentage similarity of a BGC does not signify the absence of the predicted BGC in the genome being analyzed and this correlates with our identification of iturin gene during PCR, despite its predicted similarity identity in cluster 6 (BGC0001098) being below 65%. Coding region specific for fengycin is predicted to be present in cluster 6 at 86%, bacillomycin at 66% and iturin at 53% similarity. These genes were also detected by specific primers during the PCR-gel electrophoresis and ESI-MS analysis.

Applying pan-genomic analysis helps locate genes that are responsible for species metabolic activity and genes that are dispensable to a species survival. Relying on the structural and functional metabolic capacity of well-studied or representative bacteria strains for deducing the metabolic capacity of other strains in the same genus might have limitations. Experimental data have shown that in some species new genes are discovered even after sequencing the genomes of several strains (Medini et al, 2005). *B. velezensis* sp. are categorized as heterotypic synonyms of *B. amyloliquefaciens* subsp. *plantarum* FZB42T, *B. methylotrophicus* KACC 13015T, and *B. oryzicola* KACC 18228 based on DNA-DNA hybridization values greater than 84% (Dunlap et al., 2016 and Fan et al., 2017). The *in silico* result in this study shows that our *B. velezensis* strain closely shares some phenotypic and genotypic traits with several of the commercially established plant growth promoting strains within the *Bacillus* genus.

The metabolic pathways generated in this study for BS10.5 would afford us the future bioengineering of the BS10.5 if an over expression or termination of expression is needed. Applying microbial metabolic models which are predictive pathway analysis that offer possibilities for assessing structural and functional properties of microorganisms could assist us in fully

exploiting the metabolic resources of BS10.5. It has been used as a tool for generating hypotheses and engineering the metabolism of several organisms (Patil et al., 2004; Liu et al., 2010; Zhang and Hua 2016). Microbial genome-scale metabolic models (GEMs) have been developed and used in guiding systems' metabolic engineering strategies for strain design and development (Covert et al., 2001; Oh et al., 2007; Lee et al., 2010). This strategy has also been utilized to create remarkable microbial strains for fermentative production of novel bio-based compounds, medical antibiotics, plant/crop inoculants and industrial chemicals (Zachow et al., 2015; Mienda, 2017). A recombinant *B. subtilis* strain (BBG100), obtained from a wild strain ATCC 6633 could over secrete mycosubtilin (15-fold increase) due to the manipulation of its internal mycosubtilin operon (Leclere et al., 2005).

Conclusion

Combining multiple techniques and analytical approaches for structural characterization of microbial derived compounds provides in depth information concerning the compounds being identified. The data obtained from the analysis of the BS10.5 extract suggest that surfactin, iturin and fengycin, are a few of the major antimicrobial metabolites produced by *Bacillus* BS10.5. There appears to be no standard effective chemical fungicide for the treatment of the plant diseases caused by the *Fusarium* spp. in South Africa. Based on its genomic information, *Bacillus* BS10.5 appears to be a promising BCA which could be developed as a fungicide inoculant against members of *Fusarium* spp. However, there would be need to use the bacterium or its compounds in field trials to ascertain their efficiency.

CHAPTER EIGHT

8.0 Summary, Conclusion and Recommendations

Staple crops such as rice, wheat, barley and corn are consumed globally and are equally used as starting materials for many agricultural and industrial products. Several plant pathogens do cause reduced availability of many of these crops in developed nations and scarcity of these crops are more gravely experienced in developing and underdeveloped nations. Worldwide, phytopathogens from the *Fusarium* group currently stand out among the pathogens that affect the large scale production of these cereal grains of which maize, a major and daily dietary food in South Africa is susceptible to.

In the light that an effective alleviation strategy for the control of fusariosis in South Africa is still underway, we sought to present an environmentally friendly way of managing *Fusarium graminearum* fusariosis. Many countries globally are shifting to the application of biological control measures to managing diseases that affect grain production. Plant growth promotion in its direct or indirect forms have helped in combating many of the diseases affecting maize and research involving production of native biological control products have become the current trends in many countries due to the geographical stability of indigenous microbial organisms. The existing challenge is that phytopathogens continuously present several new ways of resisting total eradication by their multiple virulent traits.

Our effort to produce a candidate biocontrol agent for the suppression of *F. graminearum* in South African maize led us to isolating rhizospheric bacterial strains from the *Pseudomonas* and *Bacillus* genera. The two genera have been researched over the years and have been established to contain species that significantly benefit man. The bacillus and pseudomonads boast of strains with genetic

capability to produce antibiotics, plant hormones and other secondary metabolites that either enhance plant growth or allow the plants to resist infectious agents. This study to the best of our knowledge provides an indigenously selected biocontrol candidate for tackling maize fusariosis, whose genomic capability is known but still underexplored.

In this study, we were able to identify from the rhizosphere of maize 7 promising biosuppressive isolates from each genera using *in vitro* approaches. Standard antifungal confrontational assays and molecular techniques assisted us in selecting these promising isolates and also detect the mechanisms they employ for their suppressive traits. Multiple antibiotic genes and genes coding for lipopeptide production were identified in some of the isolates. Two major isolates *Pseudomonas* PS9.1 (closely relate to *P. paravulva*) and *Bacillus* BS10.5 (closely related to *B. velezensis*) that exhibited distinct antifungal potential by reducing fungal mycelia development and spore formation above 50%, were further tested in green house experiments. Pre-treatment of maize grain root-seedlings with bioinoculants proved to be an effective approach to suppressing the aggressive colonization of maize by *F. graminearum*. The potential of the isolates (PS9.1 and BS10.5) to influence growth promotion of maize plant was observed during the green house experiment when treatment consisted of single bacteria strains as opposed to treatment combinations and the isolates exhibited survival traits.

During the study, a major challenge during the characterization of the BS10.5 secondary metabolites was the inability of getting purified fractions of the potent compounds. However, using spectrometric techniques we identified that the compounds surfactin, fengycin and iturin were responsible for the anti-fusarium ability of the BS10.5 isolate. The internal genomic wealth and capacity of *B. velezensis* BS10.5 could be tapped for bio-industrial applications.

Its value for agricultural purposes though in its early stages has been established, however further investigation to determine its effectiveness not only at bio-protecting crop during the germination stage against toxigenic fusarium, but also at stopping or reducing their mycotoxin levels is imperative. Field trials using inoculants made from BS10.5 or its compounds, mutagenic test to understand the roles and functions of its biosynthetic genes and exploitation of its many metabolic pathways would additional enlighten us of its other cryptic benefits. Lastly, comparative studies to determine the survivability and effectiveness of the indigenous microbial strains as opposed to imported commercial strains maybe be needed.

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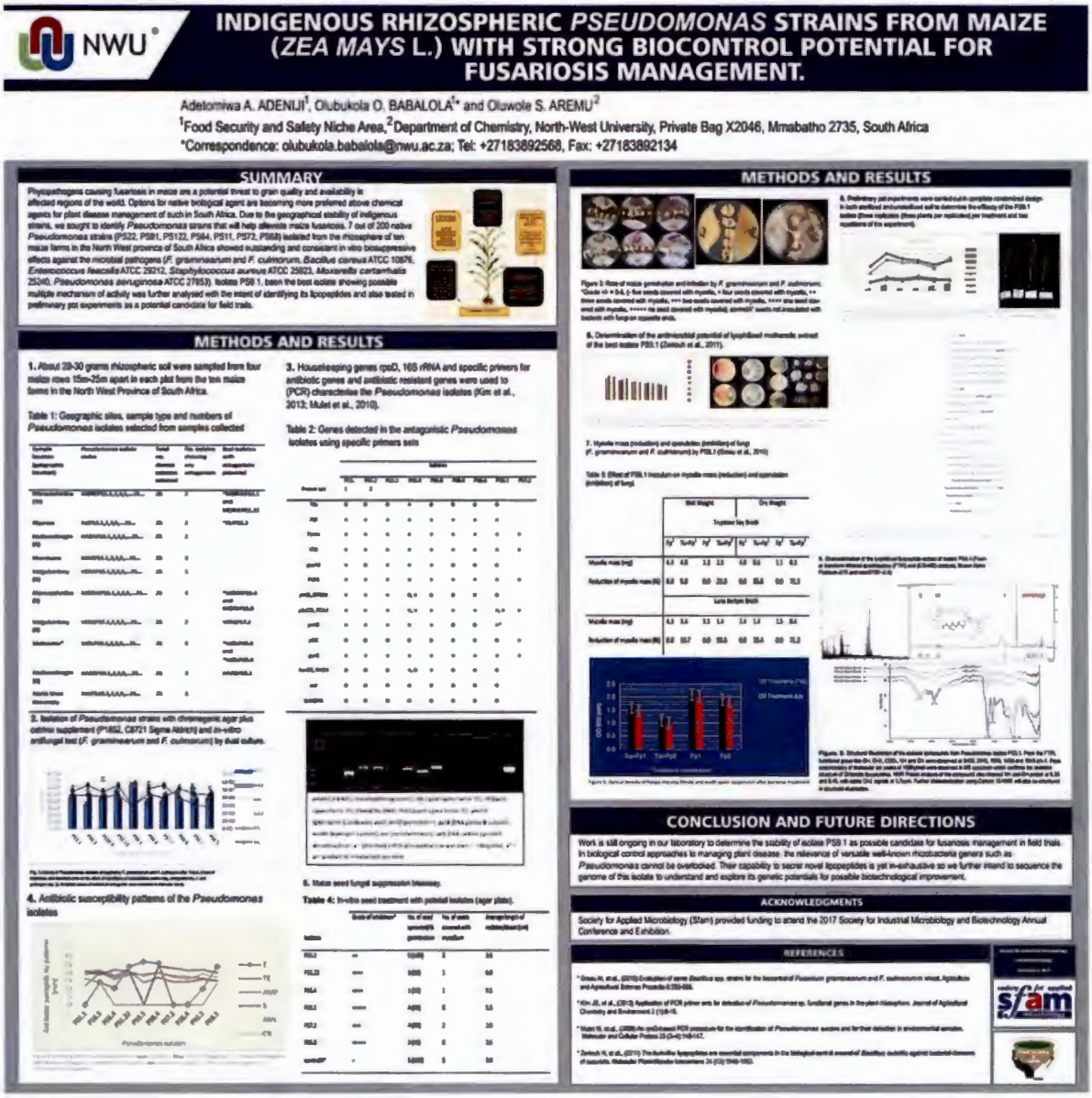
APPENDICES

Appendix 1: Excerpt of research work presented at the SIMB conference 2017



INDIGENOUS RHIZOSPHERIC *PSEUDOMONAS* STRAINS FROM MAIZE (*ZEA MAYS* L.) WITH STRONG BIOCONTROL POTENTIAL FOR FUSARIOSIS MANAGEMENT.

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SUMMARY

Phytopathogens causing fusariosis in maize are a potential threat to grain quality and availability in affected regions of the world. Options for native biological agent are becoming more preferred above chemical agents for plant disease management of such in South Africa. Due to the geographical stability of indigenous strains, we sought to identify *Pseudomonas* strains that will help alleviate maize fusariosis. 7 out of 200 native *Pseudomonas* strains (PS22, PS81, PS122, PS64, PS11, PS72, PS68) isolated from the rhizosphere of ten maize farms in the North West province of South Africa showed outstanding and consistent in vitro biosuppressive effects against the microbial pathogens (*F. graminearum* and *F. culmorum*, *Bacillus cereus* ATCC 10876, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Moraxella cartarrhalis* 25240, *Pseudomonas aeruginosa* ATCC 27853). Isolate PS81, been the best isolate showing possible multiple mechanism of activity was further analysed with the intent of identifying its lipopeptides and also tested in preliminary pot experiments as a potential candidate for field trials.

METHODS AND RESULTS

1. About 28-30 grams rhizospheric soil were sampled from four maize rows 15m×25m apart in each plot from the ten maize farms in the North West Province of South Africa.

Table 1: Geographic sites, sample type and numbers of *Pseudomonas* isolates selected from samples collected

3. Housekeeping genes rpoD, 16S rRNA and specific primers for antibiotic genes and antibiotic resistant genes were used to (PCR) characterise the *Pseudomonas* isolates (Kim et al., 2013; Mulet et al., 2010).

Table 2: Genes detected in the antagonistic *Pseudomonas* isolates using specific primers sets

2. Isolation of *Pseudomonas* strains with chromogenic agar plus cetrimi supplement (P1852, C8721 Sigma Aldrich) and *in-vitro* antifungal test (*F. graminearum* and *F. culmorum*) by dual culture.

6. Maize seed fungal suppression bioassay.

Table 4: In-vitro seed treatment with potential isolates (agar plate).

4. Antibiotic susceptibility patterns of the *Pseudomonas* isolates

METHODS AND RESULTS

Figure 3: Rate of maize germination and inhibition by *F. graminearum* and *F. culmorum*.

5. Determination of the antimicrobial potential of lyophilised methanolic extract of the best isolate PS81.1 (Zerbsch et al., 2011).

7. Mycelia mass (reduction) and sporulation (inhibition) of fungi (*F. graminearum* and *F. culmorum*) by PS81.1 (Dowa et al., 2010)

Table 5: Effect of PS81.1 inoculum on mycelia mass (reduction) and sporulation (inhibition) of fungi.

CONCLUSION AND FUTURE DIRECTIONS

Work is still ongoing in our laboratory to determine the stability of isolate PS81.1 as possible candidate for fusariosis management in field trials. In biological control approaches to managing plant disease, the relevance of versatile well-known rhizobacteria genera such as *Pseudomonas* cannot be overlooked. Their capability to secret novel lipopeptides is yet in-exhaustive so we further intend to sequence the genome of this isolate to understand and explore its genetic potentials for possible biotechnological improvement.

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Society for Applied Microbiology (Sfam) provided funding to attend the 2017 Society for Industrial Microbiology and Biotechnology Annual Conference and Exhibition.

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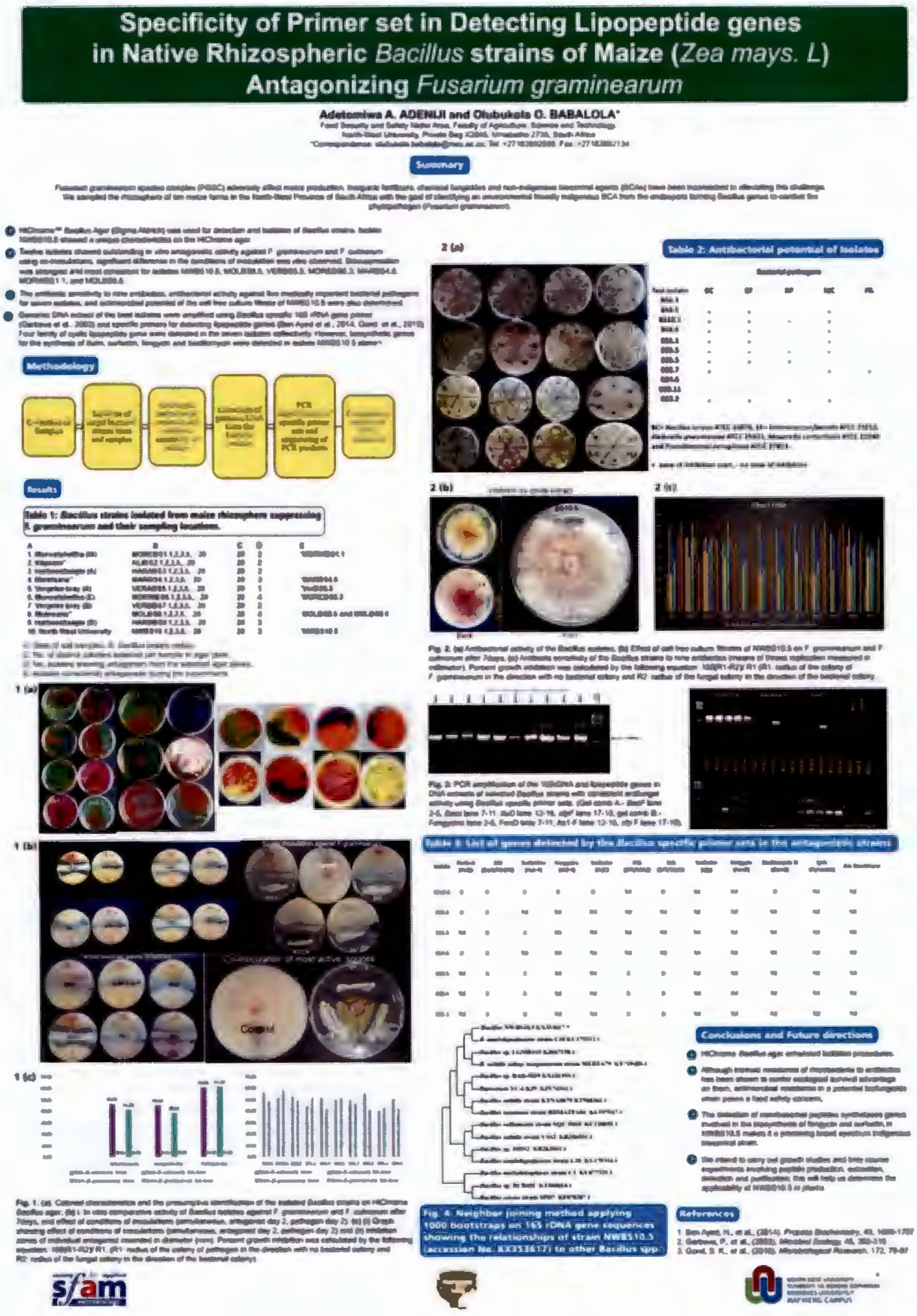
Appendix 2: Excerpt of research work presented at the Sfam conference 2016


Specificity of Primer set in Detecting Lipopeptide genes in Native Rhizospheric Bacillus strains of Maize (Zea mays. L) Antagonizing Fusarium graminearum
Adetomiwa A. ADENIJI and Olubukola O. BABALOLA*
Summary
Methodology
Results
Table 1: Bacillus strains isolated from maize rhizosphere suppressing F. graminearum and their sampling locations.
Table 2: Antibacterial potential of isolates
Table 3: List of genes detected by the Bacillus specific primer sets in the antagonistic strains
Conclusions and Future directions
References
Fig. 4. Neighbor joining method applying 1000 bootstraps on 16S rDNA gene sequences showing the relationships of strain NWBS10.5 to other Bacillus spp.

Appendix 3: Metabolic pathways of BS10.5 predicted from PATRIC

Pathway ID	Pathway Name	Pathway Class	Annotation	Unique Genome Count	Unique Gene	Unique EC Count	EC Conservation	Gene Conservation
480	Glutathione metabolism	Metabolism of Other Amino Acids	PATRIC	1	15	10	100	1.5
380	Tryptophan metabolism	Amino Acid Metabolism	PATRIC	1	35	14	100	2.5
630	Glyoxylate and dicarboxylate metabolism	Carbohydrate Metabolism	PATRIC	1	28	21	100	1.33
680	Methane metabolism	Energy Metabolism	PATRIC	1	35	19	100	1.84
51	Fructose and mannose metabolism	Carbohydrate Metabolism	PATRIC	1	43	15	100	2.87
53	Ascorbate and aldarate metabolism	Carbohydrate Metabolism	PATRIC	1	38	10	100	3.8
565	Ether lipid metabolism	Lipid Metabolism	PATRIC	1	4	2	100	2
600	Sphingolipid metabolism	Lipid Metabolism	PATRIC	1	11	5	100	2.2
730	Thiamine metabolism	Metabolism of Cofactors and Vitamins	PATRIC	1	15	13	100	1.15
740	Riboflavin metabolism	Metabolism of Cofactors and Vitamins	PATRIC	1	8	10	100	0.8
760	Nicotinate and nicotinamide metabolism	Metabolism of Cofactors and Vitamins	PATRIC	1	16	13	100	1.23
561	Glycerolipid metabolism	Lipid Metabolism	PATRIC	1	17	9	100	1.89
564	Glycerophospholipid metabolism	Lipid Metabolism	PATRIC	1	20	13	100	1.54
10	Glycolysis / Gluconeogenesis	Carbohydrate Metabolism	PATRIC	1	56	21	100	2.67
30	Pentose phosphate pathway	Carbohydrate Metabolism	PATRIC	1	25	23	100	1.09
52	Galactose metabolism	Carbohydrate Metabolism	PATRIC	1	39	15	100	2.6
230	Purine metabolism	Nucleotide Metabolism	PATRIC	1	78	53	100	1.47
500	Starch and sucrose metabolism	Carbohydrate Metabolism	PATRIC	1	42	18	100	2.33
520	Amino sugar and nucleotide sugar metabolism	Carbohydrate Metabolism	PATRIC	1	64	31	100	2.06
521	Streptomycin biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	12	9	100	1.33
627	1,4-Dichlorobenzene degradation	Xenobiotics Biodegradation and Metabolism	PATRIC	1	20	10	100	2
790	Folate biosynthesis	Metabolism of Cofactors and Vitamins	PATRIC	1	22	16	100	1.38
20	Citrate cycle (TCA cycle)	Carbohydrate Metabolism	PATRIC	1	21	14	100	1.5
270	Cysteine and methionine metabolism	Amino Acid Metabolism	PATRIC	1	41	32	100	1.28
300	Lysine biosynthesis	Amino Acid Metabolism	PATRIC	1	24	15	100	1.6
350	Tyrosine metabolism	Amino Acid Metabolism	PATRIC	1	24	10	100	2.4
360	Phenylalanine metabolism	Amino Acid Metabolism	PATRIC	1	30	15	100	2
400	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino Acid Metabolism	PATRIC	1	23	19	100	1.21
401	Novobiocin biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	6	4	100	1.5
950	Isoquinoline alkaloid biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	19	7	100	2.71
960	Tropane, piperidine and pyridine alkaloid biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	9	5	100	1.8
250	Alanine, aspartate and glutamate metabolism	Amino Acid Metabolism	PATRIC	1	32	21	100	1.52
330	Arginine and proline metabolism	Amino Acid Metabolism	PATRIC	1	55	35	100	1.57
710	Carbon fixation in photosynthetic organisms	Energy Metabolism	PATRIC	1	17	12	100	1.42
240	Pyrimidine metabolism	Nucleotide Metabolism	PATRIC	1	55	32	100	1.72
260	Glycine, serine and threonine metabolism	Amino Acid Metabolism	PATRIC	1	45	30	100	1.5
364	Fluorobenzoate degradation	Xenobiotics Biodegradation and Metabolism	PATRIC	1	2	1	100	2
930	Caprolactam degradation	Xenobiotics Biodegradation and Metabolism	PATRIC	1	8	5	100	1.6
310	Lysine degradation	Amino Acid Metabolism	PATRIC	1	21	11	100	1.91
472	D-Arginine and D-ornithine metabolism	Metabolism of Other Amino Acids	PATRIC	1	1	1	100	1

522	Biosynthesis of 12-, 14- and 16-membered macrolides	Biosynthesis of Polyketides and Nonribosomal Peptides	PATRIC	1	7	2	100	3.5
540	Lipopolysaccharide biosynthesis	Glycan Biosynthesis and Metabolism	PATRIC	1	8	3	100	2.67
563	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	Glycan Biosynthesis and Metabolism	PATRIC	1	4	1	100	4
601	Glycosphingolipid biosynthesis - lacto and neolacto series	Glycan Biosynthesis and Metabolism	PATRIC	1	5	2	100	2.5
603	Glycosphingolipid biosynthesis - globo series	Glycan Biosynthesis and Metabolism	PATRIC	1	6	3	100	2
604	Glycosphingolipid biosynthesis - ganglio series	Glycan Biosynthesis and Metabolism	PATRIC	1	9	4	100	2.25
908	Zeatin biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	5	2	100	2.5
941	Flavonoid biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	8	3	100	2.67
942	Anthocyanin biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	10	3	100	3.33
944	Flavone and flavonol biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	4	1	100	4
945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	11	4	100	2.75
965	Betalain biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	11	3	100	3.67
1051	Biosynthesis of ansamycins	Biosynthesis of Polyketides and Nonribosomal Peptides	PATRIC	1	9	4	100	2.25
1057	Biosynthesis of type II polyketide products	Biosynthesis of Polyketides and Nonribosomal Peptides	PATRIC	1	17	6	100	2.83
190	Oxidative phosphorylation	Energy Metabolism	PATRIC	1	19	5	100	3.8
40	Pentose and glucuronate interconversions	Carbohydrate Metabolism	PATRIC	1	29	22	100	1.32
643	Styrene degradation	Xenobiotics Biodegradation and Metabolism	PATRIC	1	3	3	100	1
770	Pantothenate and CoA biosynthesis	Metabolism of Cofactors and Vitamins	PATRIC	1	23	15	100	1.53
4070	Phosphatidylinositol signaling system	Signal Transduction	PATRIC	1	4	3	100	1.33
471	D-Glutamine and D-glutamate metabolism	Metabolism of Other Amino Acids	PATRIC	1	5	4	100	1.25
130	Ubiquinone and other terpenoid-quinone biosynthesis	Metabolism of Cofactors and Vitamins	PATRIC	1	21	13	100	1.62
361	gamma-Hexachlorocyclohexane degradation	Xenobiotics Biodegradation and Metabolism	PATRIC	1	5	4	100	1.25
253	Tetracycline biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	13	5	100	2.6
340	Histidine metabolism	Amino Acid Metabolism	PATRIC	1	24	17	100	1.41
626	Naphthalene and anthracene degradation	Xenobiotics Biodegradation and Metabolism	PATRIC	1	16	6	100	2.67
904	Diterpenoid biosynthesis	Biosynthesis of Secondary Metabolites	PATRIC	1	4	2	100	2

Appendix 4: Genome features of BS10.5 annotated by PATRIC

Genome	PATRIC ID	Feature Type	Start	End	Length	Strand	FIGfam ID	PATRIC genus-specific families (PLfams)	AA Length	Product
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.160	CDS	155072	156220	1149	-	FIG01230769	PLF_1386_00006186	382	Subtilisin E (EC 3.4.21.62)
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.207	CDS	199395	200135	741	+	FIG00000928	PLF_1386_00019938	246	Phytoene synthase (EC 2.5.1.32)
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.568	CDS	525818	530581	4764	+	FIG00001074	PLF_1386_00122208	1587	Polyketide synthase modules and related proteins
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.570	CDS	539327	546322	6996	+	FIG00000319	PLF_1386_00065171	2331	Polyketide synthase modules and related proteins
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.572	CDS	552057	559439	7383	+	FIG00001074	PLF_1386_00125079	2460	Modular polyketide synthase
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.588	CDS	576075	577640	1566	-	FIG00011224	PLF_1386_00007021	521	Bacillolysin, extracellular neutral metalloprotease (EC 3.4.24.28)
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.648	CDS	633464	637759	4296	+	FIG01227854	PLF_1386_00005927	1431	Bacillopeptidase F precursor (EC 3.4.21.-)
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.682	CDS	669682	670170	489	+	FIG00002381	PLF_1386_00012042	152	Siroheme synthase / Precorrin-2 oxidase (EC 1.3.1.76)
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.739	CDS	725898	726287	390	+	FIG00133479	PLF_1386_00005955	129	Flagellar basal-body rod protein FlgB
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.740	CDS	726287	726739	453	+	FIG00000587	PLF_1386_00112514	150	Flagellar basal-body rod protein FlgC
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.741	CDS	726750	727070	321	+	FIG00078127	PLF_1386_00004540	106	Flagellar hook-basal body complex protein FliE
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.742	CDS	727113	728723	1611	+	FIG00017343	PLF_1386_00004235	536	Flagellar M-ring protein FliF
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.743	CDS	728736	729752	1017	+	FIG00000610	PLF_1386_00003722	338	Flagellar motor switch protein FliG
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.744	CDS	729745	730500	756	+	FIG00000850	PLF_1386_00007213	251	Flagellar assembly protein FliH
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.745	CDS	730497	731813	1317	+	FIG01955945	PLF_1386_00003518	438	Flagellum-specific ATP synthase FliI
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.746	CDS	731819	732262	444	+	FIG00001147	PLF_1386_00006285	147	Flagellar protein FliJ
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.747	CDS	732274	732888	615	+	FIG00077673	PLF_1386_00007296	204	Flagellar protein FlbB
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.748	CDS	732895	734238	1344	+	FIG00001074	PLF_1386_00007143	447	Flagellar hook-length control protein FliK
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.749	CDS	734239	734679	441	+	FIG00000646	PLF_1386_00006629	146	Flagellar basal-body rod modification protein FlgD
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.750	CDS	734704	735480	777	+	FIG00119690	PLF_1386_00003548	258	Flagellar hook protein FlgE
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.751	CDS	735520	735735	216	+	FIG00125327	PLF_1386_00140931	71	Flagellar protein FlbD
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.752	CDS	735732	736151	420	+	FIG00057432	PLF_1386_00022390	139	Flagellar biosynthesis protein FliL
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.753	CDS	736185	737183	999	+	FIG01290464	PLF_1386_00003862	332	Flagellar motor switch protein FliM
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.754	CDS	737173	738309	1137	+	FIG00002041	PLF_1386_00003823	378	Flagellar motor switch protein FliN
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.757	CDS	739359	740024	666	+	FIG00138353	PLF_1386_00003594	221	Flagellar biosynthesis protein FliP
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.758	CDS	740039	740308	270	+	FIG00108124	PLF_1386_00003735	89	Flagellar biosynthesis protein FliQ
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.759	CDS	740315	741094	780	+	FIG00000586	PLF_1386_00022966	259	Flagellar biosynthesis protein FliR
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.760	CDS	741091	742173	1083	+	FIG00003476	PLF_1386_00003990	360	Flagellar biosynthesis protein FlhB
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.761	CDS	742207	744240	2034	+	FIG00029709	PLF_1386_00003656	677	Flagellar biosynthesis protein FlhA
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.762	CDS	744240	745331	1092	+	FIG00000950	PLF_1386_00004157	363	Flagellar biosynthesis protein FlhF
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.763	CDS	745328	746221	894	+	FIG00001155	PLF_1386_00061008	297	Flagellar synthesis regulator FleN
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.869	CDS	908969	910234	1266	+	FIG00774347	PLF_1386_00000247	421	Aluminum resistance protein
Bacillus amyloliquefaciens BS10.5	fig|1390.384.peg.904	CDS	937224	937844	621	+	FIG00002614	PLF_1386_00000425	206	Chitin binding protein

Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.3966	CDS	14769	21065	6297	-	FIG01290002	PLF_1386_00052304	2098 Polyketide synthase modules and related proteins
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.3967	CDS	21084	23720	2637	-	FIG01959596	PLF_1386_00020756	878 Modular polyketide synthase
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4011	CDS	2	2764	2763	-	FIG00018699	PLF_1386_00040274	921 Plipastatin synthase subunit D
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4012	CDS	2780	10477	7698	-	FIG00018699	PLF_1386_00136275	2565 Plipastatin synthase subunit D
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4014	CDS	1	4458	4458	+	FIG00018699	PLF_1386_00012513	1485 Plipastatin synthase subunit D
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4015	CDS	4484	10330	5847	+	FIG00018699	PLF_1386_00011067	1949 Plipastatin synthase subunit D
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4017	CDS	1884	9707	7824	+	FIG00018699	PLF_1386_00009663	2608 surfactin production and competence
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4016	CDS	3	1862	1860	+	FIG00018699	PLF_1386_00011104	619 surfactin production and competence
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4019	CDS	1	1101	1101	+	FIG01959596	PLF_1386_00016535	367 Modular polyketide synthase
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4028	CDS	3	581	579	-	FIG00018699	PLF_1386_00012513	193 Plipastatin synthase subunit D
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4032	CDS	1	561	561	-	FIG00018699	PLF_1386_00010349	187 surfactin production and competence
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4045	CDS	1	435	435	+	FIG00018699	PLF_1386_00009663	145 surfactin production and competence
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4061	CDS	3	368	366	+	FIG00018699	PLF_1386_00008697	122 surfactin production and competence
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4062	CDS	3	368	366	+	FIG00018699	PLF_1386_00010349	122 surfactin production and competence
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4065	CDS	1	360	360	+	FIG00018699	PLF_1386_00011067	120 Plipastatin synthase subunit D
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4068	CDS	2	349	348	+	FIG00118333		116 Hypothetical protein PvdY @ Siderophore synthetase small component, acetyltransferase
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4080	CDS	1	330	330	+	FIG00638284		110 Flagellum-specific ATP synthase FliI
Bacillus amyloliquefaciens FZB42	fig|1390.384.peg.4093	CDS	3	296	294	+	FIG00001914		97 Metallo-beta-lactamase superfamily protein PA0057

Appendix 5: Ortholog-MCL gene features of the BS10.5 Pangenome

Feature ID	Aliases	Genome	Type	Function
ybdZ	ybdZ	BS10.5GENOMEvelezensis	gene	Enterobactin biosynthesis protein YbdZ
ydjF_1	ydjF_1	BS10.5GENOMEvelezensis	gene	Phage shock protein A
ymfD	ymfD	BS10.5GENOMEvelezensis	gene	Bacillibactin exporter
yneB	yneB	BS10.5GENOMEvelezensis	gene	Resolvase YneB
yngHB	yngHB	BS10.5GENOMEvelezensis	gene	Biotin/lipoyl attachment protein
yokJ	yokJ	BS10.5GENOMEvelezensis	gene	Antitoxin YokJ
ypfD	ypfD	BS10.5GENOMEvelezensis	gene	30S ribosomal protein S1
ytnP	ytnP, 3.1.1.-	BS10.5GENOMEvelezensis	gene	putative quorum-quenching lactonase YtnP
ytpP	ytpP	BS10.5GENOMEvelezensis	gene	Thioredoxin-like protein YtpP
yueD	yueD, 1.1.1.320	BS10.5GENOMEvelezensis	gene	Benzil reductase ((S)-benzoin forming)

Feature ID	Aliases	Genome	Type	Function
qacA	qacA	BS10.5GENOMEvelezensis	gene	Antiseptic resistance protein
speG	speG, 2.3.1.57	BS10.5GENOMEvelezensis	gene	Spermidine N(1)-acetyltransferase
spxA	spxA	BS10.5GENOMEvelezensis	gene	Regulatory protein Spx
sqhC	sqhC, 4.2.1.137	BS10.5GENOMEvelezensis	gene	Sporulenol synthase
srfAA	srfAA	BS10.5GENOMEvelezensis	gene	Surfactin synthase subunit 1
srfAB	srfAB	BS10.5GENOMEvelezensis	gene	Surfactin synthase subunit 2
srfAC	srfAC	BS10.5GENOMEvelezensis	gene	Surfactin synthase subunit 3
sttH	sttH, 3.5.2.19	BS10.5GENOMEvelezensis	gene	Streptothricin hydrolase
tlyA	tlyA	BS10.5GENOMEvelezensis	gene	Hemolysin A
tlyC	tlyC	BS10.5GENOMEvelezensis	gene	Hemolysin C

Feature ID	Aliases	Genome	Type	Function
entB	entB, 6.3.2.14	BS10.5GENOMEvelezensis	gene	Enterobactin synthase component B
groL	groL	BS10.5GENOMEvelezensis	gene	60 kDa chaperonin
HCFCLKJD_00450	None	BS10.5GENOMEvelezensis	gene	Flavodoxin
HCFCLKJD_00452	None	BS10.5GENOMEvelezensis	gene	Flavodoxin
HCFCLKJD_00647	None	BS10.5GENOMEvelezensis	gene	tRNA-Val(gac)
HCFCLKJD_00849	None	BS10.5GENOMEvelezensis	gene	Small, acid-soluble spore protein 2
HCFCLKJD_00985	None	BS10.5GENOMEvelezensis	gene	ESAT-6-like protein
HCFCLKJD_01280	None	BS10.5GENOMEvelezensis	gene	Gluconeogenesis factor
HCFCLKJD_01787	None	BS10.5GENOMEvelezensis	gene	Desiccation/radiation resistance protein
HCFCLKJD_01878	5.1.3.11	BS10.5GENOMEvelezensis	gene	Cellobiose 2-epimerase

Feature ID	Aliases	Genome	Type	Function
murJ_1	murJ_1	BS10.5GENOMEvelezensis	gene	Lipid II flippase MurJ
ndoAI	ndoAI	BS10.5GENOMEvelezensis	gene	Antitoxin EndoAI
npr	npr, 3.4.24.28	BS10.5GENOMEvelezensis	gene	Bacillolysin
pcp	pcp, 3.4.19.3	BS10.5GENOMEvelezensis	gene	Pyrrolidone-carboxylate peptidase
pigC_1	pigC_1, 6.4.-.-	BS10.5GENOMEvelezensis	gene	Prodigiosin synthesizing transferase PigC
ppsA	ppsA, 2.3.1.-	BS10.5GENOMEvelezensis	gene	Plipastatin synthase subunit A
ppsB	ppsB, 2.3.1.-	BS10.5GENOMEvelezensis	gene	Plipastatin synthase subunit B
ppsC	ppsC, 2.3.1.-	BS10.5GENOMEvelezensis	gene	Plipastatin synthase subunit C
ppsD	ppsD, 2.3.1.-	BS10.5GENOMEvelezensis	gene	Plipastatin synthase subunit D
ppsE	ppsE, 2.3.1.-	BS10.5GENOMEvelezensis	gene	Plipastatin synthase subunit E

Feature ID	Aliases	Genome	Type	Function
HCFCLKJD_03278	None	BS10.5GENOMEvelezensis	gene	Antimicrobial peptide LCI
HCFCLKJD_03295	None	BS10.5GENOMEvelezensis	gene	TelA-like protein
hemC	hemC, 2.5.1.61	BS10.5GENOMEvelezensis	gene	Porphobilinogen deaminase
kipA	kipA	BS10.5GENOMEvelezensis	gene	KipI antagonist
levS	levS, 2.4.1.10	BS10.5GENOMEvelezensis	gene	Levansucrase
lrgB	lrgB	BS10.5GENOMEvelezensis	gene	Antiholin-like protein LrgB
moaD	moaD	BS10.5GENOMEvelezensis	gene	Molybdopterin synthase sulfur carrier subunit
mrsA	mrsA	BS10.5GENOMEvelezensis	gene	Lantibiotic mersacidin
mrsD	mrsD, 4.1.1.-	BS10.5GENOMEvelezensis	gene	Mersacidin decarboxylase
mshD_1	mshD_1, 2.3.1.189	BS10.5GENOMEvelezensis	gene	Mycothiol acetyltransferase

Appendix 6: Multivariate analysis of the anti-pathogenic activity of the lyophilized extract and commercial fungicides on fungal spores.

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.838	25.883[b]	2.000	10.000	.000
	Wilks' Lambda	.162	25.883[b]	2.000	10.000	.000
	Hotelling's Trace	5.177	25.883[b]	2.000	10.000	.000
	Roy's Largest Root	5.177	25.883[b]	2.000	10.000	.000
Treatment	Pillai's Trace	.422	.736	8.000	22.000	.659
	Wilks' Lambda	.621	.672[b]	8.000	20.000	.710
	Hotelling's Trace	.539	.607	8.000	18.000	.761
	Roy's Largest Root	.319	.876[c]	4.000	11.000	.509

a. Design: Intercept + Treatment; b. Exact statistic; c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Appendix 7: Multivariate test of mycelial mass reduction and spore suppressing capacity of rhizobacteria isolates in different media

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	889926.084[b]	3.000	28.000	.000
	Wilks' Lambda	.000	889926.084[b]	3.000	28.000	.000
	Hotelling's Trace	95349.223	889926.084[b]	3.000	28.000	.000
	Roy's Largest Root	95349.223	889926.084[b]	3.000	28.000	.000
Treatment	Pillai's Trace	2.770	24.141	45.000	90.000	.000
	Wilks' Lambda	.000	534.038	45.000	83.961	.000
	Hotelling's Trace	65867.800	39032.771	45.000	80.000	.000
	Roy's Largest Root	65799.766	131599.532[c]	15.000	30.000	.000
Rep	Pillai's Trace	.278	1.564	6.000	58.000	.174
	Wilks' Lambda	.738	1.528[b]	6.000	56.000	.186
	Hotelling's Trace	.331	1.491	6.000	54.000	.199
	Roy's Largest Root	.233	2.252[c]	3.000	29.000	.103

a. Design: Intercept + Treatment + Rep; b. Exact statistic; c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Appendix 8: Analysis of variance of the effect of bacterial treatments on seed-borne incidence of *F. graminearum* on maize.

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.999	7495.462[b]	6.000	34.000	.000
	Wilks' Lambda	.001	7495.462[b]	6.000	34.000	.000
	Hotelling's Trace	1322.729	7495.462[b]	6.000	34.000	.000
	Roy's Largest Root	1322.729	7495.462[b]	6.000	34.000	.000
Treatment	Pillai's Trace	4.541	9.341	78.000	234.000	.000
	Wilks' Lambda	.000	19.024	78.000	193.572	.000
	Hotelling's Trace	124.269	51.513	78.000	194.000	.000
	Roy's Largest Root	103.029	309.088[c]	13.000	39.000	.000
Rep	Pillai's Trace	.376	.859	18.000	108.000	.628
	Wilks' Lambda	.658	.857	18.000	96.652	.629
	Hotelling's Trace	.471	.855	18.000	98.000	.633
	Roy's Largest Root	.339	2.033[c]	6.000	36.000	.086

a. Design: Intercept + Treatment + Rep; b. Exact statistic; c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Appendix 9: Multivariate test of the second harvest: Effect of bacterial treatments on seed-borne incidence of *F. graminearum* on maize.

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.997	3406.402[b]	4.000	45.000	.000
	Wilks' Lambda	.003	3406.402[b]	4.000	45.000	.000
	Hotelling's Trace	302.791	3406.402[b]	4.000	45.000	.000
	Roy's Largest Root	302.791	3406.402[b]	4.000	45.000	.000
Treatment	Pillai's Trace	3.027	9.956	60.000	192.000	.000
	Wilks' Lambda	.000	18.296	60.000	177.885	.000
	Hotelling's Trace	64.533	46.786	60.000	174.000	.000
	Roy's Largest Root	56.830	181.856[c]	15.000	48.000	.000

Appendix 10: Multivariate test of the first harvest: Effect of bacterial treatments on seed-borne incidence of *F. graminearum* on maize.

Multivariate Tests[a]

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	20790.162[b]	4.000	27.000	.000
	Wilks' Lambda	.000	20790.162[b]	4.000	27.000	.000
	Hotelling's Trace	3080.024	20790.162[b]	4.000	27.000	.000
	Roy's Largest Root	3080.024	20790.162[b]	4.000	27.000	.000
Treatment	Pillai's Trace	3.529	15.000	60.000	120.000	.000
	Wilks' Lambda	.000	35.180	60.000	107.622	.000
	Hotelling's Trace	173.298	73.652	60.000	102.000	.000
	Roy's Largest Root	134.801	269.603[c]	15.000	30.000	.000
Rep	Pillai's Trace	.730	4.028	8.000	56.000	.001
	Wilks' Lambda	.359	4.510[b]	8.000	54.000	.000
	Hotelling's Trace	1.532	4.980	8.000	52.000	.000
	Roy's Largest Root	1.347	9.427[c]	4.000	28.000	.000

Appendix 11: Multivariate analysis of antibiotics sensitivity of *Bacillus* (A) and *Pseudomonas* (B) isolates

Multivariate Tests A

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	.999	711.209[b]	11.000	8.000	.000
	Wilks' Lambda	.001	711.209[b]	11.000	8.000	.000
	Hotelling's Trace	977.913	711.209[b]	11.000	8.000	.000
	Roy's Largest Root	977.913	711.209[b]	11.000	8.000	.000
Treatments	Pillai's Trace	5.362	2.772	88.000	120.000	.000
	Wilks' Lambda	.000	2.683	88.000	61.919	.000
	Hotelling's Trace	26.308	1.868	88.000	50.000	.009
	Roy's Largest Root	8.275	11.284[c]	11.000	15.000	.000

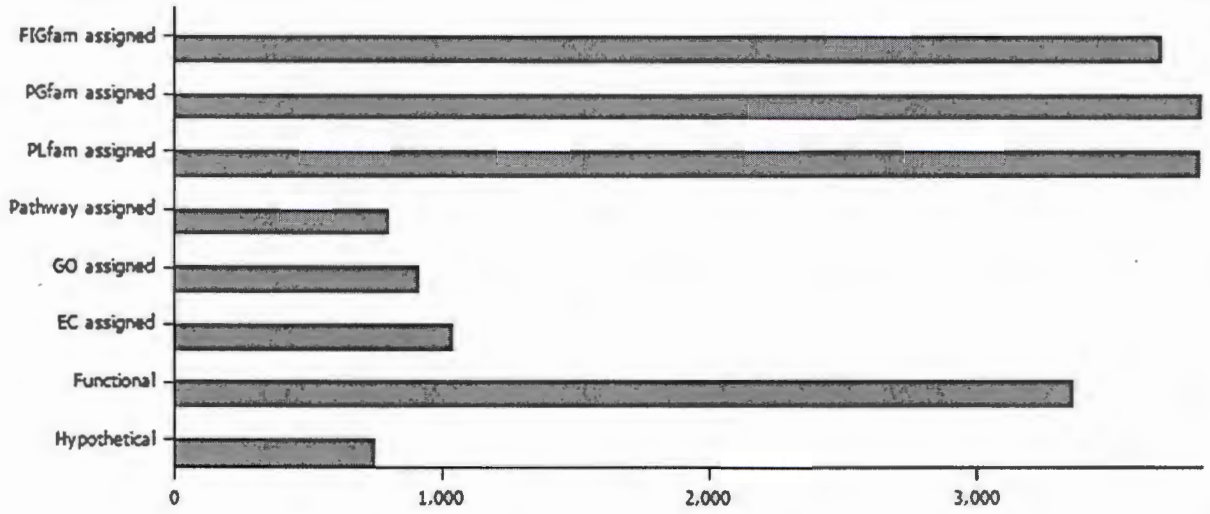
Multivariate Tests B

Effect		Value	F	Hypothesis df	Error df	Sig.
Intercept	Pillai's Trace	1.000	1710703.652[b]	11.000	4.000	.000
	Wilks' Lambda	.000	1710703.652[b]	11.000	4.000	.000
	Hotelling's Trace	4704435.043	1710703.652[b]	11.000	4.000	.000
	Roy's Largest Root	4704435.043	1710703.652[b]	11.000	4.000	.000
Treatment	Pillai's Trace	5.988	412.890	66.000	54.000	.000
	Wilks' Lambda	.000	8899.817	66.000	26.859	.000
	Hotelling's Trace	800551.349	28302.320	66.000	14.000	.000
	Roy's Largest Root	748086.608	612070.861[c]	11.000	9.000	.000

a. Design: Intercept + Treatments; b. Exact statistic; c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Appendix 12: PATRIC BS10.5 protein features

Protein Features

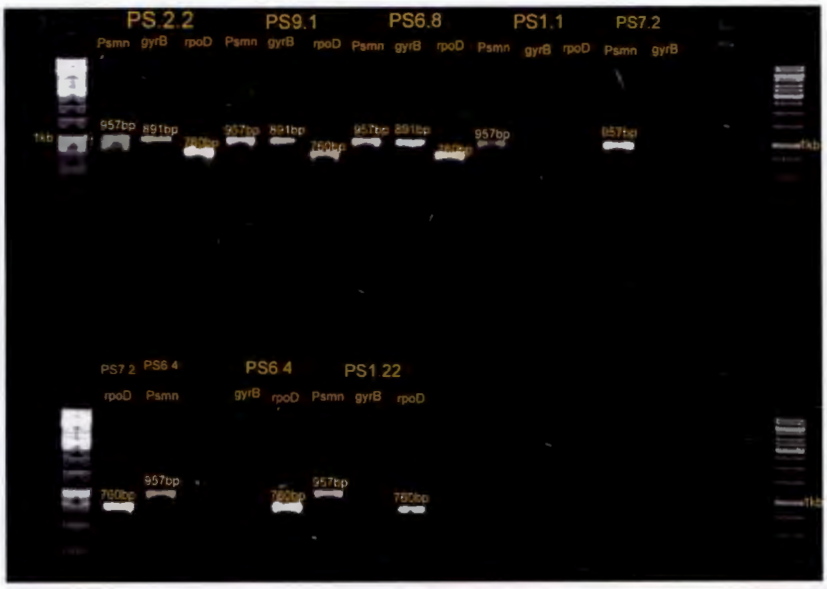


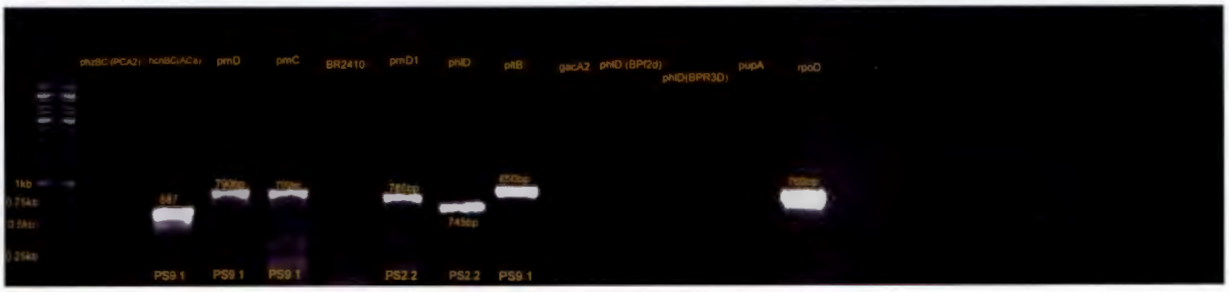
Protein Features

	PATRIC	RefSeq
Hypothetical proteins	744	0
Proteins with functional assignments	3355	0
Proteins with EC number assignments	1038	0
Proteins with GO assignments	907	0
Proteins with Pathway assignments	799	0
Proteins with PATRIC genus-specific family (PLfam) assignments	3831	0
Proteins with PATRIC cross-genus family (PGfam) assignments	3844	0
Proteins with FIGfam assignments	3685	0

Specialty Genes

Appendix 12: Agarose gel photograph showing amplicons of functional genes in selected *Pseudomonas* strains with consistent antifungal activity. PCR amplification by degenerate primers

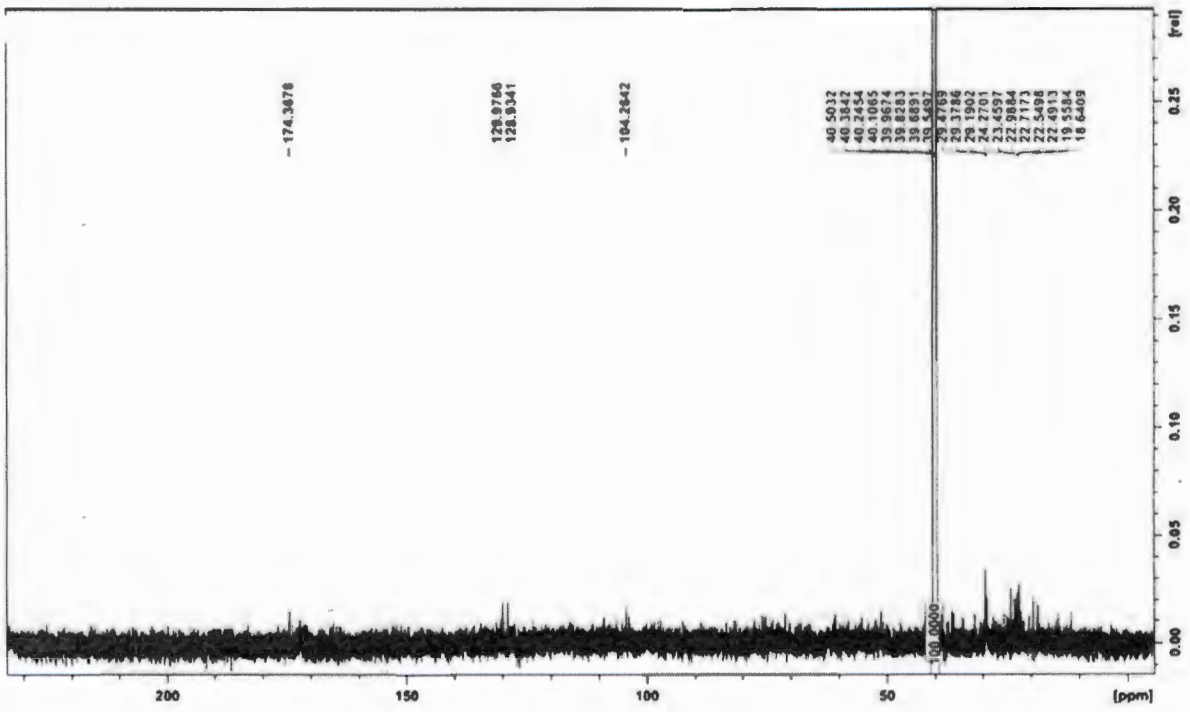




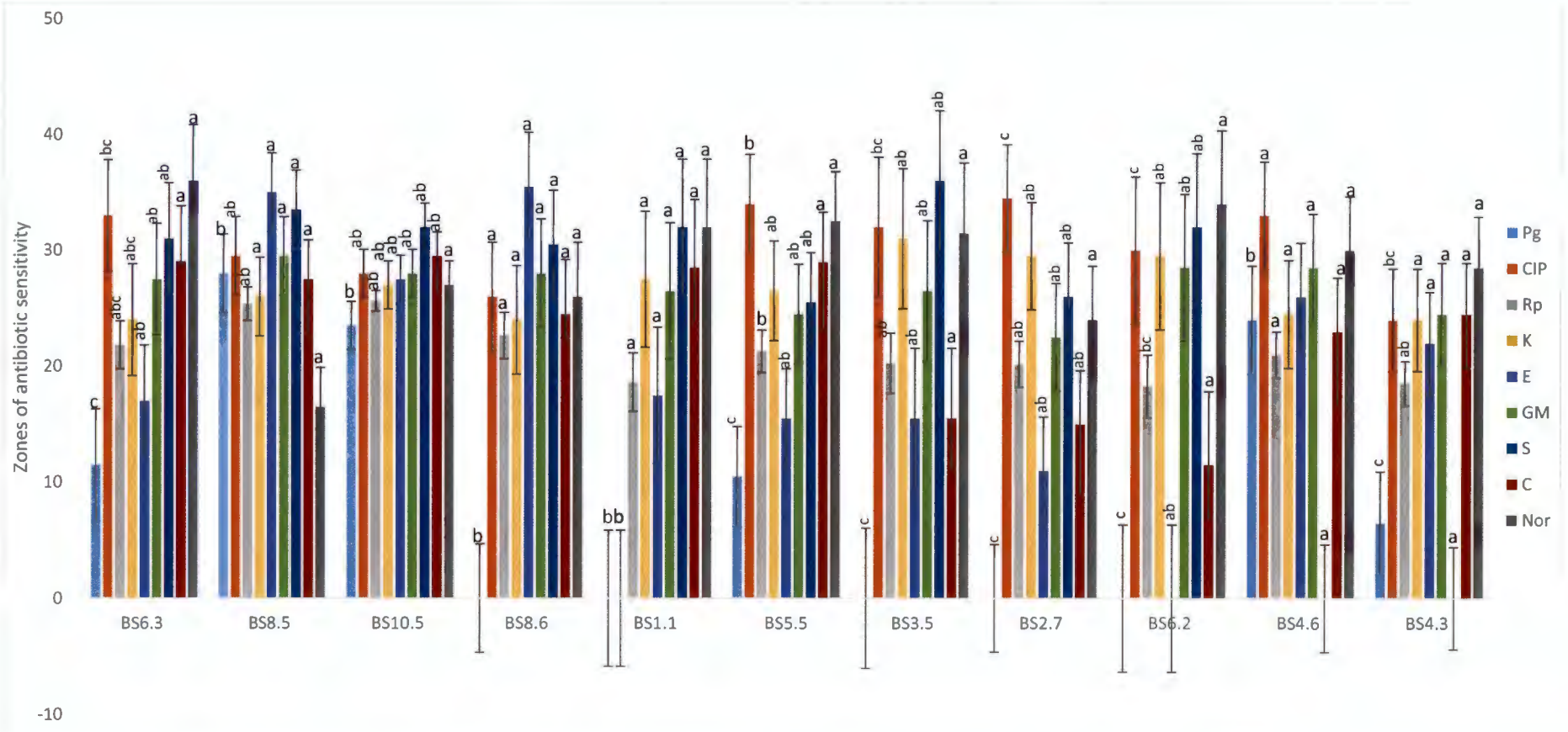
Appendix 13: BS10.5 subsystem comparison with other selected *Bacillus* genome subsystem

Similar FIG Sequence	E-value	Percent Identity	Aligned Positions of Query [?]	Aligned Positions of Hit [?]	Organism [?]	Function [?]	Associated Subsystem	Evidence Code [?]	FIGfam
Query Sequence: **fig|1390.385.peg.3**	n/a	n/a	n/a	n/a	**Bacillus amyloliquefaciens BS10.5**	**Plipastatin synthase subunit D**	**None added**		○
fig|224308.1.peg.1836	0.0	64.84%	1-1180 **(1180/1180)**	**1375-2555 (1181/2555)**	Bacillus subtilis subsp. subtilis str. 168	PHENOLPTHIOCEROL SYNTHESIS TYPE-I POLYKETIDE SYNTHASE PPSC	None added	cwn dlit(10471562)	○
fig|1390.385.peg.3812	3e-30	48.31%	629-745 **(117/1180)**	2-119 **(118/123)**	**Bacillus amyloliquefaciens BS10.5**	surfactin production and competence	None added		○
fig|1390.385.peg.2414	0.0	45.33%	1-1177 **(1177/1180)**	1-1191 **(1191/1194)**	**Bacillus amyloliquefaciens BS10.5**	Plipastatin synthase subunit D	None added		○
fig|243365.1.peg.2803	2e-132	44.52%	105-696 **(592/1180)**	**2315-2134 (305/3171)**	Chromobacterium violaceum ATCC 12472	probable peptide synthetase protein	None added	cwn	○
fig|224308.1.peg.1835	0.0	44.11%	1-1177 **(1177/1180)**	**2405-3600 (1196/3603)**	Bacillus subtilis subsp. subtilis str. 168	peptide synthetase	None added	cwn dlit(10471562)	○
fig|391612.3.peg.5538	3e-148	43.36%	8-674 **(667/1180)**	554-1227 **(674/1245)**	Cyanothece sp CCY 0110	**Thioesterase domains of type I polyketide synthases or non-ribosomal peptide synthetases**	None added		○
fig|1390.385.peg.2310	0.0	43.27%	2-1180 **(1179/1180)**	**1377-2565 (1189/2565)**	**Bacillus amyloliquefaciens BS10.5**	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	None added		○
fig|1390.385.peg.2415	9e-151	43.14%	50-696 **(647/1180)**	389-1033 **(645/1267)**	**Bacillus amyloliquefaciens BS10.5**	FIG01239039: hypothetical protein	None added		○
fig|171440.1.peg.2101	4e-127	42.81%	101-706 **(606/1180)**	1330-1943 **(614/5457)**	Photorhabdus asymbiotica subsp. asymbiotica	**Peptide synthetase**	None added		○
fig|1390.385.peg.1427	7e-135	42.09%	606-1173 **(568/1180)**	1-566 **(566/570)**	**Bacillus amyloliquefaciens BS10.5**	surfactin production and competence	None added		○
fig|1390.385.peg.3768	1e-128	42.07%	606-1179 **(574/1180)**	1-572 **(572/575)**	**Bacillus amyloliquefaciens BS10.5**	surfactin production and competence	None added		○
fig|243365.1.peg.2802	2e-115	41.98%	105-696 **(592/1180)**	**2679-3277 (599/3554)**	Chromobacterium violaceum ATCC 12472	probable peptide synthetase protein	None added	cwn	○
fig|431943.4.peg.1777	2e-153	41.37%	1-703 **(703/1180)**	1097-1803 **(707/2005)**	Clostridium kluyveri DSM 555	Putative polyketide synthase	None added		○
fig|224308.1.peg.1837	0.0	41.18%	2-1180 **(1179/1180)**	1374-2560 **(1187/2560)**	Bacillus subtilis subsp. subtilis str. 168	Phenolpthiocerol synthesis polyketide synthase ppsB	None added	cwn dlit(10471562)	○
fig|1390.385.peg.2309	0.0	40.77%	1-1180 **(1180/1180)**	10-1197 **(1188/1197)**	Bacillus amyloliquefaciens BS10.5	Malonyl CoA-acyl carrier protein transacylase (EC 2.3.1.39)	None added		○
fig|224308.1.peg.349	0.0	40.68%	6-1173 **(1168/1180)**	2420-3584 **(1165/3588)**	Bacillus subtilis subsp. subtilis str. 168	surfactin production and competence	None added	cwn dlit(1847909)	○
fig|1390.385.peg.2442	0.0	40.61%	16-1180 **(1165/1180)**	144-1322 **(1179/2619)**	**Bacillus amyloliquefaciens BS10.5**	Siderophore biosynthesis non-ribosomal peptide synthetase modules	None added		○
fig|1390.385.peg.2441	0.0	40.57%	22-1173 **(1152/1180)**	1672-2814 **(1143/5365)**	**Bacillus amyloliquefaciens BS10.5**	Siderophore biosynthesis non-ribosomal peptide synthetase modules	None added		○
fig|1390.385.peg.2413	1e-92	40.51%	149-614 **(466/1180)**	11-471 **(461/471)**	**Bacillus amyloliquefaciens BS10.5**	surfactin production and competence	None added		○
fig|1390.385.peg.3874	1e-61	39.31%	479-814 **(336/1180)**	1-336 **(336/337)**	**Bacillus amyloliquefaciens BS10.5**	Plipastatin synthase subunit D	None added		○
fig|452637.4.peg.2050	9e-124	39.17%	1-700 **(700/1180)**	**2937-3593 (716/3631)**	Opitutus terrae PB90-1	pyoverdine synthetase D	None added		○
fig|1390.385.peg.3767	4e-135	39.16%	48-703 **(656/1180)**	384-1043 **(660/1278)**	Bacillus amyloliquefaciens BS10.5	Siderophore biosynthesis non-ribosomal peptide synthetase modules	None added		○
fig|452637.4.peg.2054	0.0	39.09%	1-1166 **(1166/1180)**	355-1502 **(1148/3018)**	Opitutus terrae PB90-1	non-ribosomal peptide synthetase	None added	cwn	○

Appendix 14: NMR carbon spectrum of BS10.5



Appendix 15



Supplementary fig. 3.1: Susceptibility patterns of *Bacillus* isolates to varying antibiotic concentrations. Values are means and standard error of three replicates; bars tagged with same letters at the top indicates values that are not significantly different according to Duncan's least significant difference test at $P \leq 0.05$.

Appendix 16: Susceptibility patterns of *Bacillus* isolates to varying antibiotic concentrations.

Test isolate	Pg	CIP	Rp	K	E	GM	S	C	NOR
BS6.3	17	39	17	30	23	33	37	35	42
BS8.5	34	34	25	32	41	34	39	33	23
BS10.5	28	34	27	32	34	34	38	34	33
BS8.6	0	32	25	30	41	34	36	30	32
BS1.1	0	0	22	33	23	33	38	34	38
BS5.5	16	40	18	32	20	30	31	34	38
BS3.5	0	38	17	37	21	32	42	21	37
BS2.7	0	40	27	35	17	28	32	21	30
BS3.2	0	36	19	34	0	34	38	17	40
BS4.6	30	38	21	29	32	34	0	29	36
BS4.3	11	30	25	30	28	30	0	29	34

Appendix 17

Supplementary table 4.1a: Percentage inhibition of *F. culmorum* mycelia by *Bacillus* isolates

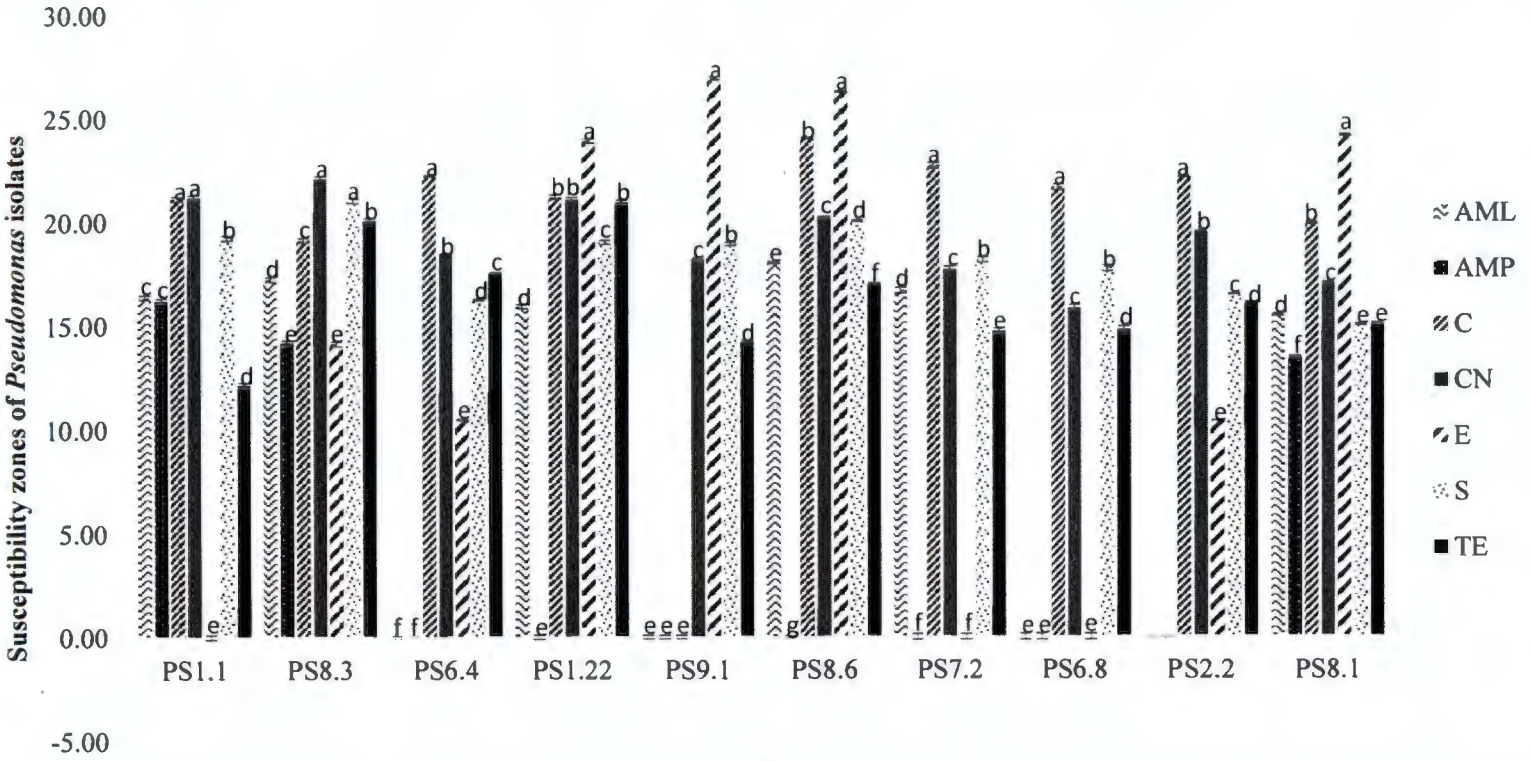
	Condition (% inhibition zone)			
Treatment	1	2	3	mean
PS1.1	48.00	56.00	42.67	48.89
PS1.22	45.33	52.00	44.00	47.11
PS10.4	50.67	41.33	41.33	44.44
PS2.2	46.67	57.33	45.33	49.78
PS6.8	44.00	62.67	52.00	52.89
PS7.2	52.00	49.33	40.00	47.11
PS8.1	54.67	50.00	44.00	49.56
PS8.3	52.00	54.67	44.00	50.22
PS8.6	49.33	56.00	45.33	50.22
PS9.1	68.00	38.67	29.33	45.33
PS1.1	48.00	56.00	42.67	48.89
mean	51.067a	51.455	42.800a	
ANOVA				
Treatment (T)	***			
Condition (C)	***			
T x C	***			

Appendix 18

Supplementary table 4.1b: Percentage inhibition of *F. graminearum* mycelia by *Pseudomonas* isolates

	Condition (% inhibition zone)			
Treatment	1	2	3	mean
PS1.1	46.67	61.33	42.67	52.00
PS1.22	56.00	58.67	44.00	56.00
PS10.4	45.33	73.33	41.33	56.44
PS2.2	62.67	44.00	45.33	47.11
PS6.8	54.67	57.33	52.00	51.56
PS7.2	41.33	62.67	40.00	54.22
PS8.1	45.33	60.00	44.00	45.33
PS8.3	36.00	65.33	44.00	52.44
PS8.6	41.33	66.67	45.33	54.67
PS9.1	65.33	62.67	29.33	52.44
mean	49.467a	61.091	46.000a	
ANOVA				
Treatment (T)	***			
Condition (C)	***			
T x C	***			

Appendix 19



Supplementary fig. 4.2: Susceptibility patterns of *Pseudomonas* isolates to varying antibiotic concentrations. Values are means and standard error of three replicates; bars tagged with same letters at the top indicates values that are not significantly different according to Duncan's least significant difference test at $P \leq 0.05$.

Appendix 20: Susceptibility patterns of *Pseudomonas* isolates to varying antibiotic concentrations.

Isolates	E	TE	AMP	S	AML	CN	C
PS1.1	0	12	16.5	19	16	21	21
PS8.3	14	20	14	21	17	22	19
PS6.4	10.5	17.5	0	16	0	18.5	22
PS1.22	24	21	0	19	16	21	21
PS9.1	27	14	0	19	0	18	0
PS8.6	26	17	0	20	18	20	24
PS7.2	0	14.5	0	18	16.5	17.5	23
PS6.8	0	14.5	0	17.5	0	16	21.5
PS2.2	10	16	0	16.5	0	19.5	22
PS8.1	24	15	13.5	15	15.5	17	20